FECAL MICROBIOME IN DOGS WITH ACUTE DIARRHEA

A Thesis

by

BLAKE CROSBY GUARD

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Chair of Committee,	Jan S. Suchodolski
Committee Members,	Jörg M. Steiner
	James W. Barr
	Arul Jayaraman
Head of Department,	Sandee M. Hartsfield

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ABSTRACT

Recent molecular studies have revealed that the canine gastrointestinal tract (GIT) harbors a highly complex microbial ecosystem. Gut microbes play a very important role in the development and regulation of the immune system of the host, mediated in-part through the production of immunomodulatory metabolites (e.g., butyrate, propionate, indole). Limited information is available about potential changes in the predominant bacterial groups in dogs with acute diarrhea, and characterizing the functional gene content of the microbiome may help to understand relationships between microbiota, endogenous metabolites, and gastrointestinal disease. Therefore, the aim of this study was (1) to characterize the fecal microbiome in healthy dogs, dogs with acute non-hemorrhagic diarrhea (NHD), and dogs with acute hemorrhagic diarrhea (AHD) using 16S rRNA gene sequencing and qPCR analysis; (2) to measure fecal concentrations of short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs); and (3) to describe the functional gene content of the fecal microbiome.

Fecal samples were collected from healthy dogs (n=13), dogs with NHD (n=5), and dogs with AHD (n=6). The fecal microbiota were analyzed by 454-pyrosequencing of 16S rRNA genes and qPCR assays. SCFAs were quantified by gas chromatography/mass spectrometry (GC/MS). Functional genes present in the microbiome were predicted from the 16S rRNA gene data using the software PICRUSt.

The Shannon Index for bacterial diversity was significantly decreased in dogs with acute diarrhea (AD; both NHD and AHD groups combined) compared to healthy

dogs ($p=0.0020$). Sequences belonging to Bacteroidetes were significantly decreased in dogs with AD compared to healthy dogs ($p=0.0280$). Sequences belonging to the genus *Faecalibacterium* and an unclassified genus within the family Ruminococcaceae were both significantly decreased in dogs with AD compared to healthy dogs ($p=0.0319$ and 0.0368, respectively). Also, a significant decrease in *Blautia* spp. were observed in dogs with AD compared to healthy dogs ($p=0.0472$). The proportions of butyric acid were significantly increased and proportions of propionic acid were significantly decreased in dogs with AD compared to healthy dogs ($p<0.05$ for both). Significant differences were not observed in functional categories among all dogs after adjustment for multiple comparisons.

Results of this study revealed a bacterial dysbiosis in fecal samples of dogs with NHD and dogs with AHD compared to healthy dogs. The bacterial groups that were commonly decreased during acute diarrhea are considered to be important SCFA producers and may be important for canine intestinal health. Future studies to evaluate broader metabolomic profiles in dogs with acute diarrhea are indicated.

DEDICATION

To Melissa and my family.

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NOMENCLATURE

NHD	acute non-hemorrhagic diarrhea
AHD	acute hemorrhagic diarrhea
AD	acute diarrhea (both NHD and AHD)
GIT	gastrointestinal tract
GI	gastrointestinal
qPCR	quantitative real-time polymerase chain reaction
spp.	species
unclass.	unclassified taxa
SCFAs	short-chain fatty acids
BCFAs	branched-chain fatty acids
PCoA	principle coordinate analysis
LEfSe	linear discriminant analysis effect size
ANOSIM	analysis of similarity
OTUs	observational taxonomic units
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG ortholog

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

MICROBIOTA IN GASTROINTESTINAL HEALTH AND IMMUNITY

It is estimated that the human intestinal tract contains up to 100 trillion microbes. The entire gene content of this collection of microbes is referred to as the intestinal microbiome, and it is thought to contain 100 times more genes than the human genome [1,2]. Also, the canine gastrointestinal tract harbors a complex and highly diverse assembly of bacteria [3-5]. Given this complex and intrinsic bacterial population, it is not surprising that there is an inherent relationship between host and resident microbiota that is important for gastrointestinal health [6]. Gastrointestinal microbes stimulate the gastrointestinal immune system and the postnatal development of gut structure. Furthermore, microbes aid in defending against invading pathogens and produce various metabolites (e.g., short-chain fatty acids (SCFAs), vitamins) that can be utilized by the host [7]. The intestinal microbiota also plays a crucial role in intestinal immunity. For example, intestinal bacteria are recognized by toll-like receptors (TLRs), and these TLRs play a key role in the innate defense by communicating with the immune system to activate adaptive lymphoid tissue [8]. Studies of the gastrointestinal microbiota in germ-free animals have revealed insight into the development of host phenotype [9]. The gastrointestinal tract can be highly dynamic and must readily adapt, especially during the neonatal period [9]. In the neonate, physiological microbial colonization of the gastrointestinal tract is believed to advance critical systems responsible for humoral and

cellular mucosal immunity as well as normal host physiology [9]. This is evident from studies involving germ-free rodent models. These germ-free animals, by definition born and raised with a total lack of the intestinal microbiota, typically require an increased energy intake and have lower serum concentrations of vitamins B and K [9,10]. Furthermore, they have an altered intestinal structure and morphology and display delayed gastric motility compared to conventionally raised animals [10,11].

Given the importance of intestinal microbes for gastrointestinal health, studies have evaluated various factors that influence the composition and activity of the gastrointestinal microbiota. Of particular interest is the effect of various diets on fecal bacterial populations. One study evaluated the effects of a high-fiber diet (10% total fiber; 5% soybean hulls and 5% beet pulp) compared to a control diet low in fiber on the fecal microbiota of healthy dogs [12]. No significant changes in fecal concentrations of various microbial metabolites (e.g., ammonia, sulfide, and indole) were observed in the dogs fed the 10% high-fiber diet [12]. In contrast, feeding the high-fiber diet led to significant increases in fecal concentrations of the SCFAs acetate, propionate, and butyrate compared to the low-fiber diet. However, no changes in fecal microbial populations were observed in dogs fed the high-fiber diet compared to those in dogs fed the low-fiber control diet [12]. A separate study compared the effects of three different diets: a high-carbohydrate diet (starch concentration: 438 g/kg), a high-protein diet (crude protein concentration: 609 g/kg), and a standard commercial dry diet (starch and crude protein concentration: 277 g/kg and 264 g/kg; respectively) on the colonic microbiota of healthy Beagle dogs [13]. The fecal microbiota in dogs fed the dry

commercial diet generally harbored increased bacterial abundances of Clostridiales, Lactobacillales, Coriobacteriales, and Bacteroidales. Sequences belonging to Clostridiales were the most common bacterial order in fecal samples from dogs fed the dry commercial diet and high-carbohydrate diet. Overall, the proportion of Clostridiales in dogs fed the high-protein diet was significantly decreased compared to dogs fed the dry commercial diet. Lactobacillales and Bacteroidales were not detected in fecal samples from dogs fed the high-carbohydrate and high-protein diets [13]. These studies show that the fecal microbiota of dogs can be altered by changes in the diet.

CHARACTERIZATION OF THE INTESTINAL MICROBIOTA

Bacteria are the most predominant microbes present in the GI tract of mammals, comprising approximately >98% of microbial cells [14]. In the past, studies have focused primarily on cultivation methods to characterize the bacterial groups present in the GI tract. Studies in humans and dogs reported that the predominant culturable bacterial groups in the GI tract were *Lactobacillus*, *Bifidobacterium*, *Eubacterium*, *Bacteroides*, and *Peptostreptococcus* spp. [15,16]. Cultivation techniques, however, may not provide a complete picture of the gastrointestinal microbiota; the GI tract harbors many anaerobic bacteria, which are subject to degradation during sample handling. Therefore, these anaerobes are often poorly represented in the results of cultivation studies [17-19]. Because bacterial culture underestimates microbial diversity, molecular tools typically targeting the 16S ribosomal ribonucleic acid (rRNA) gene, are now being commonly utilized and allow to characterize a more complete profile of the

gastrointestinal microbiota [19-22]. In one study, the bacterial phyla Firmicutes (47.7%), Proteobacteria (23.3%), Fusobacteria (16.6%), and Bacteroidetes (12.4%) were the major bacterial groups identified in intestinal content collected from the duodenum, jejunum, ileum, and colon of six healthy dogs [23]. Other bacterial phyla such as Spirochaetes, Tenericutes, Verrucomicrobia, TM7, Cyanobacteria, Chloroflexi, Planctomycetes, and some unclassified bacterial lineages generally make up lesser proportions (< 1%) of bacteria throughout the gastrointestinal (GI) tract in healthy dogs [24]. DNA sequence analysis of the human fecal microbiota has revealed somewhat similar bacterial proportions, with Firmicutes (81.2%), Actinobacteria (14.6%), Bacteroidetes (2.5%), and Proteobacteria (1.7%), representing the highest percentage of sequence reads in one study [25]. Similarly, based on one study, the fecal microbiota of mice is dominated by Firmicutes (56.2%), Bacteroidetes (24.4%), Actinobacteria (15.5%), and Proteobacteria (3.0%) [26].

Culture-based methods. There are inherent limitations to cultivation techniques when attempting to characterize complex microbial ecosystems such as the ones residing in the GI tract of mammals [27]. Some authors have suggested that more than 99% of all environmental prokaryotes are uncultivable [27], and studies estimate that only 10-50% of bacteria that are part of the gastrointestinal microbiota can be cultured [14,17,22,27,28]. Limitations of bacterial culture include a lack of optimal growth conditions for the majority of gastrointestinal microorganisms and the need for anaerobic handling of isolated bacteria [22]. Mutualistic interactions between the various microorganisms and/or the host that are required for optimal growth of bacteria can often

not be well replicated *in vitro* [29]. Lastly, classification of bacterial species based on phenotypic (e.g., assessment of the morphology of isolated colonies) is often inaccurate, and many cultured isolates require molecular typing for accurate identification of isolates [20,30].

Molecular-based methods. Since bacterial culture largely underestimates the microbial diversity in the GI tract, molecular approaches are now commonly being used to characterize gut microbial ecology. Molecular tools require DNA or RNA to be extracted from biological samples (e.g., feces, biopsy specimens, or luminal content). Specific genes (e.g., the 16S rRNA gene) are then amplified using universal bacterial primers and the diversity of the bacterial populations are evaluated using phylogenetic methods such as DNA sequencing. Molecular-based methods allow demonstration of evolutionary relationships between bacterial communities and between samples, while identifying the presence and abundance of bacteria in a given sample [31].

While molecular-based methods are currently more frequently employed for the characterization of bacterial communities, the techniques involved in 16S rRNA gene sequencing are not new. In 1977 Sanger et al. developed a sequencing method, which utilized chain-terminating inhibitors of DNA polymerase and was the most rapid and accurate method at the time [32]. This method was, however, costly and initial studies characterizing the gut microbiota using clone libraries were limited to a few hundred sequences per sample [33]. Over time, there have been vast improvements for robustness and cost-effectiveness in 16S rRNA gene sequencing [32]. For example, 454-pyrosequencing is an automated high-throughput sequencing platform that is capable of

sequencing several thousand PCR amplicons within a few hours [29]. Newer platforms in sequencing such as Illumina and Ion-Torrent have recently been introduced and are even more-cost effective than 454-pyrosequencing [34]. Since the development of high-throughput methods, similar advances in processing of the large amounts of sequencing data generated by these automated platforms have become necessary. Open-source software tools such as QIIME which stands for Quantitative Insights into Microbial Ecology are now available [35]. QIIME allows phylogenetic analysis of 16S rRNA gene sequences and provides an analysis pipeline that reaches from the raw sequencing output created by the sequencing machine through bacterial taxonomy assignment, statistical analysis, and graphical visualization of microbial communities [35].

For the purposes of this introduction, quantitative PCR and pyrosequencing reaction methodologies are outlined to describe the primary tools that were utilized in this research project:

Polymerase Chain Reaction (PCR). Conventional PCR relies upon electrophoresis-based visualization of target amplicons at the end of the assay after all amplification cycles have been performed. In contrast, quantitative real-time PCR (qPCR) monitors the progress of the amplification reaction as it occurs in real time. With qPCR, a larger amount of starting material (DNA or cDNA) is directly proportional to fluorescence emission [36]. Cyanine dye (e.g., SYBR® Green I) and hydrolysis probe (e.g., TaqMan®) based assays are two major sequence detection chemistries employed in qPCR assays.

SYBR® Green I is a dye that binds to double stranded DNA and the fluorescence signal increases proportional to the generation of double stranded PCR product [37]. SYBR® Green I dye chemistry does not require a probe, and is more cost effective compared to hydrolysis probe based assays. This method however, can generate false positive signals, as SYBR® Green I dye indiscriminately binds to any double stranded DNA present in a reaction tube, including primer dimers and also non-specific amplification products [38].

DNA and cDNA can also be quantified by TaqMan® chemistry [37]. In a TaqMan® assay, an oligonucleotide probe is designed containing a reporter fluorescent dye (e.g., 6-carboxyfluorescein [FAM] or tetrachlorofluorescein [TET]) on the 5' end and a quencher dye (e.g., tetramethylrhodamine [TAMRA]) on the 3' end of the probe. The quencher dye acts to reduce the fluorescence emitted by the reporter dye when in solution. If the target sequence is present, the probe anneals downstream of the primer and is subsequently cleaved by the 5' nuclease activity of Taq DNA polymerase after extension of the primer. Once the probe is cleaved, the reporter dye is released and separated from the quencher dye resulting in a measurable increase in fluorescence signal. TaqMan® chemistry is highly specific for the target sequence; it is also possible to target two distinct sequence targets in one reaction tube if different reporter dyes are used. A limitation of TaqMan® chemistry is that different probes must be synthesized for different sequences in addition to forward and reverse primers, which makes these assays more expensive than SYBR® Green assays [39,40].

Pyrosequencing reaction. First, an amplicon is generated by PCR that contains a biotinylated 5' terminus. After linking biotinylated double-stranded DNA, amplicons are then linked to a solid surface coated with streptavidin and denatured. During DNA synthesis and successful incorporation of nucleotides, inorganic pyrophosphate (PPi) is consequentially generated and detected by a camera and visualized on a computer, then translated into nucleotide bases.

Biochemically, a nucleic acid polymerization reaction takes place, which leads to the release of PPi that is then incorporated into adenosine triphosphate (ATP) by ATP sulfurylase. Generated ATP is then detected by luciferase-producing photons. Luciferase in turn oxidizes luciferin, generating light. Any unused ATP and deoxynucleotide is then degraded by the nucleotide-degrading enzyme apyrase to reduce interference during PPi detection. In a traditional pyrosequencing machine, an inkjet cartridge is used to distribute enzymes, substrates, and all sequential nucleotides into the wells of a microtiter plate. The plate undergoes continuous agitation to increase reaction efficiency [41]. A lens array is used for detecting fluorescence from each well projected onto the chip of a camera.

Limitations of molecular methods. While molecular methods are becoming widely used, it is important to recognize that these methods also have limitations. Different cell lysis procedures during DNA extraction can introduce inter-assay variability (e.g., bead beating vs. heating of lysis buffer) [24,42]. Another limitation is that 16S rRNA gene copies cannot be directly converted into cell counts because some bacteria can have multiple numbers of rRNA operons [5]. Operons can vary from 1 to 15

within each individual bacterial phylotype and are subject to change throughout cell growth and metabolic activity. Therefore, the abundance of bacterial groups with higher operon counts may be more likely to be falsely overestimated [43].

THE CANINE INTESTINAL MICROBIOTA

The gastrointestinal tract of dogs is inhabited by a diverse and complex microbiota; our understanding of the composition and dynamics of it is very basic but growing [42]. In one study, bacterial abundances were investigated in various regions of the GI tract of nine healthy male Beagle dogs [16]. The highest number of bacteria cultured was 10^{10} colony-forming units (CFU) per gram of tissue and colonic content. Throughout the GIT the most abundant genera were *Bacteroides*, *Bifidobacterium*, *Peptostreptococcus*, *Eubacterium*, *Clostridium*, and *Peptococcus*. *Streptococcus* represented the highest number of facultative anaerobes. Microscopic counting of bacterial cell suspensions using a Petroff-Hausser (PH) chamber revealed that cecal and ileal compartments harbor about one to three less logs of CFUs per gram of tissue and contents than in the colon [16].

One study reported bacterial abundances in the jejunal content and feces from twenty-two Beagle dogs [44]. In this study, small intestinal fluid samples as well as naturally passed feces were analyzed [44]. The microbiota of seven of these dogs was evaluated at weekly time intervals over a four week period [44]. Aerobic and facultative anaerobic bacteria on one side and anaerobic bacteria on the other were found with equal abundance in jejunal samples, while anaerobic bacteria were significantly more abundant

in fecal samples [44]. While total bacterial numbers were significantly less abundant in the jejunum than in the feces (range: 10^2 to 10^6 CFU/g vs. 10^8 to 10^{11} CFU/g, respectively), some bacterial groups were more abundant in the proximal small intestine compared to feces [44]. *Staphylococcus* and non-fermentative gram-negative rods were found in greater proportions in the proximal small intestine than in the feces (64% versus 36% and 27% versus 9% of all bacterial organisms, respectively) [44]. Bile-resistant *Bacteroides* spp., *Clostridium hiranonis*-like organisms, and *Lactobacillus* spp. were highly abundant in fecal samples [44]. The microbiota in the small intestine was much less diverse and showed significant fluctuations over time compared to the fecal microbiota which remained stable over time [44]. These results show that the microbiota is highly diverse throughout the gastrointestinal tract [44].

One study used 16S rRNA gene sequencing to characterize the fecal bacterial and fungal communities in 12 healthy dogs and 12 healthy cats using bacterial and fungal tag-encoded FLX-Titanium pyrosequencing [4]. Additionally, group-specific 16S rRNA gene clone libraries for *Bifidobacterium* spp. and *Lactobacillus* spp. were constructed. The results showed that the most prevalent bacterial phyla in the feces of dogs were Firmicutes and Bacteroidetes [4]. Furthermore, the most prevalent bacterial class in the feces of dogs was *Clostridia*, mostly comprised of the genera *Clostridium* (clusters XIVa and XI) and *Ruminococcus* [4].

454 FLX high-throughput pyrosequencing was used to analyze the fecal microbiota of eleven healthy adult miniature Schnauzer dogs in one study [45]. The dogs were of mixed sex and age and some shared common genetic lineages. They were all

housed on the same premises and had relatively controlled exercise and feeding habits. Fusobacterium (40%), Bacteroidetes (33%), Proteobacteria (11%), and Actinobacteria (<1%) were found to be the predominant phyla in the fecal microbiota. Individual dogs showed a high variability in the abundance of bacterial genera in their fecal samples [45]. Alpha-diversity measures for species richness (e.g., Chao 1 metric; a predictive measure) showed that fecal microbiota may harbor as many as 500 to 1,500 observational taxonomic units (OTUs) [45].

Another study investigated the bacterial diversity in the lumen of the canine duodenum, jejunum, ileum, and colon by direct sequence analysis of the 16S rRNA gene [23]. Intestinal contents were collected from six healthy unrelated Hound dogs; immediately after euthanasia the abdominal cavity was opened and the intestines were isolated. Sequences belonging to Clostridiales were most abundant in the duodenum (40%) and jejunum (39%). Sequences belonging to *Clostridium* (clusters XIVa and XI) were the predominant groups in the proximal small intestine and colon. Fusobacteriales was the most abundant order found in the ileum (33%), while Bacteroidales was the most abundant order found in the colon (30%). The order Enterobacteriales was more abundant in the small intestine than in the colon, and Lactobacillales occurred widespread throughout the gastrointestinal tract [23].

THE ROLE OF THE INTESTINAL MICROBIOTA IN CANINE GASTROINTESTINAL DISEASE

A fecal dysbiosis has been reported in dogs with clinical signs of GI disease [46]. Furthermore, studies have indicated that the microbiota is actually needed for the spontaneous development of intestinal inflammation. Studies in mice with targeted deletion of the gene coding for anti-inflammatory cytokine IL-10 were used to test the hypothesis that enteric bacteria are necessary for the development of spontaneous colitis and immune system activation [47]. IL-10-deficient ($IL\text{-}10^{-/-}$) mice develop inflammation of the intestine with a resident microbial population, which can be abated by the application of IL-10 [48]. Mice that are $IL\text{-}10^{-/-}$ and kept under germ-free conditions do not develop enterocolitis, suggesting that a resident microbial community is necessary for a GI response in IL-10 deficient mice [47-52].

One study investigated the predominant fecal microbiota of dogs with chronic diarrhea by fluorescence in situ hybridization (FISH) and Denaturing Gradient Gel Electrophoresis (DGGE) and examined the effects of a fiber blend on the canine fecal microbiota [53]. Dogs with chronic diarrhea (n=9) and healthy control dogs (n=8) were fed a high-fiber regimen (i.e., psyllium, which is a fermentable fiber) for three weeks [53]. Dogs with chronic diarrhea and healthy control dogs shared the *Atopobium* cluster, the *Lactobacillus-Enterococcus* group, and the *Clostridium* cluster XIV as predominant bacterial groups [53]. Dogs with chronic diarrhea had a significantly increased cell count of *Bacteroides* at baseline and significantly decreased cell counts of *Atopobium* cluster cells following fiber supplementation compared to healthy control dogs [53]. The

abundance of *Atopobium* cluster increased significantly in healthy control dogs, while cell counts of sulphate-reducing bacteria decreased significantly. Also, *Clostridium* clusters I and II cell counts increased significantly in dogs with chronic diarrhea during fiber supplementation [53].

To further characterize the bacterial microbiota in dogs with various gastrointestinal disorders, one study analyzed fecal samples from healthy dogs (n=32), dogs with acute non-hemorrhagic diarrhea (n=12), dogs with acute hemorrhagic diarrhea (n=13), and dogs with active IBD (n=9), and therapeutically controlled idiopathic IBD (n=10) [54]. Analysis was performed by 454-pyrosequencing and qPCR assays of the 16S rRNA gene. Bacterial communities of dogs with acute hemorrhagic diarrhea and acute non-hemorrhagic diarrhea were notably different than those of healthy dogs (Analysis of Similarity (ANOSIM): p=0.001 and p=0.003; respectively) as observed by principle coordinate analysis (PCoA) plots based on unweighted UniFrac distances [54]. *Blautia* spp. and Ruminococcaceae, including the genera *Faecalibacterium* and *Turicibacter*, were significantly decreased in dogs with acute hemorrhagic diarrhea [54]. Members of the genus *Sutterella* and *Clostridium perfringens* were significantly increased in dogs with acute hemorrhagic diarrhea compared to healthy dogs. Microbial communities between healthy dogs and dogs with IBD were not significantly different as observed on PCoA plots. *Faecalibacterium* spp. and Fusobacteria decreased significantly in dogs with clinically active IBD; during remission, however, their abundances increased [54]. Also, short-chain fatty acid producing bacteria (e.g.,

Erysipelotrichaceae, Ruminococcaceae, and Lachnospiraceae) were significantly decreased in dogs with acute diarrhea [54].

Lastly, another study utilized qPCR based on the 16s rRNA gene. Feces in healthy dogs (n = 242), dogs with chronic enteropathies (CE) (n = 118), and dogs with acute hemorrhagic diarrhea (AHD) (n = 57) were evaluated [46]. There were marked differences in bacterial abundances between healthy dogs, dogs with CE, and dogs with AHD. Abundances of *Faecalibacterium* spp., *Turicibacter* spp., and Ruminococcaceae were significantly decreased in dogs with CE and AHD compared to healthy dogs. *Lactobacillus* spp. and *Streptococcus* spp. were significantly increased in dogs with CE. Abundances of *Clostridium perfringens* and *E. coli* were significantly increased in dogs with AHD compared to healthy dogs. This was also consistent for dogs with CE who had a significant increase in the abundance of *E.* coli compared to healthy control dogs. Finally, while the abundance of Bacteroidetes was significantly increased in dogs with CE, the abundance of Firmicutes was decreased in dogs with AHD when compared to healthy control dogs [46].

HYPOTHESIS AND OBJECTIVES

The hypothesis of this study was that differences exist between the fecal microbiome and fecal concentrations of short-chain-fatty-acids (SCFA) between healthy dogs, dogs with acute non-hemorrhagic diarrhea (NHD), and dogs with acute hemorrhagic diarrhea (AHD).

The objectives of this study were to

(1) evaluate the fecal microbiota in healthy dogs, dogs with NHD, and dogs with AHD using 16S rRNA gene sequencing and qPCR analysis,

(2) to predict the fecal metagenome based on the 16S rRNA gene data, and

(3) to measure fecal concentrations of short-chain fatty acids (SCFA).

CHAPTER II

FECAL MICROBIOME IN DOGS WITH ACUTE DIARRHEA

SUMMARY

The aim of this study was to evaluate the fecal microbiota in healthy dogs, dogs with acute non-hemorrhagic diarrhea (NHD), and dogs with acute hemorrhagic diarrhea (AHD) using 16S rRNA gene sequencing and quantitative real-time Polymerase Chain Reaction (qPCR) analysis, to predict the functional content from the 16S rRNA gene data, and to measure fecal concentrations of short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs). Fecal samples were collected from 13 healthy dogs, 5 dogs with NHD, and 6 dogs with AHD. For 454-pyrosequencing and qPCR, feces from 8 of 13 healthy dogs and all dogs from the diseased groups (n=11) were used. For some dogs, there were not enough left-over feces to complete all analyses therefore analysis of SCFA and BCFA consisted of fecal samples from 8 of 13 healthy dogs, 3 of 5 dogs with NHD, and 4 of 6 dogs with AHD.

Microbial diversity was significantly decreased in dogs with AD compared to healthy dogs (Shannon Index: p=0.0022). More specifically when comparing all groups (e.g., healthy dogs, dogs with NHD, and dogs with AHD), a Dunn's post-test revealed a significant decrease in microbial diversity in dogs with NHD, compared to healthy dogs (Shannon Index: p=0.0208). Fecal microbial communities were significantly different between healthy dogs and dogs with AD (ANOSIM; p=0.0040). Furthermore, microbial communities were significantly different between healthy dogs and dogs with NHD and

dogs with AHD (ANOSIM: p=0.0020 for both). Abundances of sequences belonging to the phylum Bacteroidetes were significantly decreased in dogs with AD compared to healthy dogs (p=0.0280). Also, abundances of sequences belonging to the genus *Faecalibacterium* and an unclassified genus within the family Ruminococcaceae were both significantly decreased in fecal samples from dogs with AD compared to those from healthy dogs (p=0.0319 and 0.0368, respectively). In contrast, sequences belonging to the genus *Clostridium* were significantly increased in dogs with AD compared to healthy dogs (p=0.0476). Results from qPCR revealed that the abundance of *Blautia* spp. were significantly decreased in dogs with AD compared to healthy dogs (p=0.0472), while the abundance of *Clostridium perfringens* was significantly increased in dogs with AD compared to healthy dogs (p=0.0088). When comparing all groups (e.g., healthy dogs, dogs with NHD, and dogs with AHD), a Dunn's post-test revealed a significant increase in sequences belonging to *Clostridium perfringens* in dogs with AHD compared to healthy dogs ($p<0.05$). Proportionally, propionic acid was significantly decreased in the feces from dogs with AD compared to those from healthy dogs (p=0.0033). In contrast, the proportion of fecal butyric acid was significantly increased in dogs with AD compared to healthy dogs (p=0.0048). There were no significant differences observed in the functional gene content among all dogs after correcting for multiple comparisons.

In conclusion, differences in individual bacterial populations and bacterial communities were identified between healthy dogs and dogs with acute diarrhea. Fecal SCFA concentrations were also different between healthy dogs and dogs with acute

diarrhea. Further studies are warranted to evaluate the microbiome in dogs with acute diarrhea and evaluate treatment modalities that restore microbial balance.

INTRODUCTION

Recent molecular studies have greatly increased our knowledge concerning the microbiota harbored in the GIT of dogs, mice, and humans [4,22,42,55]. It has been suggested that the total microbial load in the intestinal tract is somewhere between 10^{12}-10^{14} organisms, which is approximately 10 times the number of host cells. Studies have confirmed that the vast number of microbes that make up the GIT microbiota are a necessity in host health, as they play an important role in stimulating the immune system, development of gut structure, aiding in the defense against pathogens, and providing nutritional benefit to the host (e.g., production of SCFAs) [29,56-60]. However, the microbiota has also been implicated in the pathogenesis of certain GIT disorders, and must be studied intensely to understand their relationships with the host [29].

It has been suggested that in acute episodes of diarrhea the intestinal microbiota (e.g., enterotoxigenic *C. perfringens*, *Salmonella*, viruses, and parasites) play a key role in pathogenesis of this condition. Other GI disorders (e.g., chronic diarrhea) may also be subject to these pathogenic bacteria [54,61,62]. There is limited information from molecular studies describing the fecal microbiota in dogs with acute diarrhea and of those available, results have varied. Fluorescent *in situ* hybridization (FISH) probes were used in one study and found that *Bacteroides* cell counts were significantly increased in

Beagle dogs that had chronic diarrhea [53]. A study in the feces from dogs with unspecified diarrhea used 454-pyrosequencing of the cpn60 gene and found a significantly decreased proportion of Bacteroidetes [63]. Another study in dogs with episodes of diarrhea revealed increased abundances of *Clostridium perfringens*, *Enterococcus faecalis*, and *E. faecium* using terminal restriction fragment length polymorphism (T-RFLP) analysis and qPCR [56]. One study specifically enrolled dogs with acute hemorrhagic diarrhea and dogs with active and therapeutically controlled idiopathic IBD. Results from 454-pyrosequencing revealed that dogs with acute hemorrhagic diarrhea had significant decreases in *Blautia* spp. and Ruminococcaceae, which are both SCFA producers [54]. These studies are indicative of a fecal dysbiosis that is present in dogs with GI disease. However, high-throughput sequencing studies in dogs with well-defined disease phenotypes are limited [54].

Studies in humans that focus on GI disease have revealed bacterial shifts that significantly reduce the amount of SCFA producing bacteria [64,65]. SCFA producing bacteria in one study (e.g., *Ruminococcus* spp., *Faecalibacterium* spp., *Dorea* spp., and *Turicibacter* spp.) were found to predominate in the ileum and colon [5]. A decreased diversity of *Clostridium* clusters XIVa and IV (i.e., Ruminococcaceae*, Faecalibacterium prausnitzii*, and *C. coccoides* subgroups) in IBD patients may indicate that these SCFA producing bacteria are important in the maintenance of GI health [29,64,65]. Fermentation of dietary substrates such as starch, fiber, cellulose, pectin, and fructans by bacteria produce SCFAs, which are a preferred energy source for colonocytes and critical in metabolism and epithelial cell growth [66,67].

Recent studies have provided strong data concerning the bacterial phylogeny based on 16S rRNA gene surveys. However, these studies still do not provide any information pertaining to function. A metagenomics approach could be advantageous as it provides data about community structure [68,69]. In one study fecal samples from healthy dogs were collected and a metagenomics approach was used to characterize the effects of supplemental dietary fiber. The most represented functional categories among the sample population were carbohydrates, protein metabolism, cell wall and capsule, cofactors, vitamins, prosthetic groups and pigments, DNA metabolism, RNA metabolism, amino acids/derivatives, and virulence factors [69]. Although brief overviews of metagenomic studies have been provided, more studies are required to characterize the metagenome in healthy dogs and dogs with GI disease.

Given the progress of these inter-related fields our aim was to: further evaluate the fecal microbiota in healthy dogs, dogs with acute non-hemorrhagic diarrhea (NHD), and dogs with acute hemorrhagic diarrhea (AHD) using 16S rRNA gene sequencing and qPCR analysis, analyze functional gene content based on the 16S rRNA gene data, and measure fecal concentrations of SCFAs and BCFAs.

MATERIALS AND METHODS

Animal enrollment and sample collection. Naturally passed fecal samples were obtained from healthy dogs as well as dogs with acute diarrhea (AD); dogs with AD were further classified as dogs with acute non-hemorrhagic diarrhea (NHD) or dogs with acute hemorrhagic diarrhea (AHD) (Table 1). Feces were refrigerated immediately after

Table 1. Signalment for dogs enrolled in the study.

Animal ID	Age (years)	Breed	Sex and sexual status
H1	3	Labrador	MN
H2	7	Boston terrier mix	MN
H3	9	Labrador	FS
H4	5	German shepherd	FS
H5	3	Australian Kelpie	F
H6	1	Labrador mix	F
H7	10	Weimaraner	MN
H8	8	Miniature schnauzer	FS
H9	12	Miniature schnauzer	FS
H10	5	Boxer mix	MN
H11	3	Australian shepherd	FS
H12	1	Maltipoo	FS
H13	7	Boston terrier	MN
NHD1	1	Husky/shepherd mix	FS
NHD2	1	Miniature pinscher	F
NHD3	12	American collie	FS
NHD4	1	Brittany spaniel	M
NHD5	6	Basset hound	FS
AHD1	7	Labrador	MN
AHD2	1	Brittany spaniel	MN
AHD3	5	Bulldog	MN
AHD4	3	Shetland sheepdog	FS
AHD5	2	Dachshund	F
AHD6	10	American Pit Bull terrier	MN

H = Healthy dogs; NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea.
M = intact male; MN = male neutered; F = intact female; FS = female spayed.

collection, transferred within a few hours to a -80° C freezer, and stored frozen until processing for DNA extraction. The collection of fecal samples was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC): Protocol Number; 2012-101.

The control group consisted of 13 healthy dogs (Table 1). Since there was not enough feces available from each individual healthy dog for all analyses, fecal material from healthy animals with IDs H1 to H8 were used for 454-pyrosequencing and qPCR analysis, while feces from healthy animals with IDs H4, H7, and H8 to H13 were used in SCFA analysis, resulting in an n=8 for the healthy control group. All dogs were privately owned, lived in various home environments, and were fed a variety of commercial diets. None of the dogs had a history of gastrointestinal signs or administration of antibiotics for at least a month prior to collection of fecal samples. All healthy dogs lived in Texas, USA.

The diseased group consisted of 11 pet dogs with AD (5 NHD and 6 AHD) that presented to the Veterinary Medical Teaching Hospital at Texas A&M University with acute, non-hemorrhagic or hemorrhagic diarrhea (duration of diarrhea <3 days) (Table 1). Unfortunately, there was not enough left-over feces available from each individual dog to analyze SCFA/BCFA concentrations, therefore, 3 of 5 (animal IDs: NHD3 to NHD5) dogs with NHD and 4 of 6 (animal IDs: AHD1 to AHD2 and AHD4 to AHD6) dogs with AHD were used. None of the dogs had a previous history of GI signs or had received antibiotics within the previous three months. Diagnostic evaluation included a complete blood count (CBC), serum chemistry profiles (SIRRUS® Clinical Chemistry

Analyzer), serum concentrations of canine trypsin-like immunoreactivity (cTLI) concentration, serum concentrations of canine pancreatic lipase immunocreactivity (cPLI), serum concentrations of cobalamin and folate (Immulite 2000 Vitamin B12, Folic Acid, Siemens Medical Solutions Diagnostics), and serum concentrations of C-reactive protein (CRP; Phase ™Range Canine C-reactive Protein Assay, Tridelta Development Ltd), and urine analysis.

Summary statistics of both healthy and diseased groups of dogs are displayed in Table 2.

DNA isolation. Approximately 100 mg of feces was aliquoted into a sterile 1.7 ml tube (Microtube, Sarstedt AG & Co, Nümbrecht, Germany) containing 150 µl of 0.1 mm zirconia-silica beads and 100 µl of 0.5 mm zirconia-silica beads (BioSpec Products Inc., Barlesville, OK, USA). The DNA was extracted using the ZR fecal DNA Mini Prep kit (Zymo Research, Irvine CA, USA). A volume of 750 µl of lysis buffer was added to each sample. Samples were then homogenized (FastPrep-24, MP Biomedicals, USA) for a duration of 1 minute at a speed of 4 m/s. Tubes then underwent centrifugation at 10,000 x g for 1 minute and the remaining DNA extraction was carried out as recommended by the manufacturer. For downstream PCR analysis the concentration of the isolated DNA was measured by a spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE USA) and normalized to 5 ng/µl by adding PCR quality water.

Table 2. Basic summary statistics of all dogs.

	Healthy	NHD	AHD	AD	M-W p-value	K-W p-value
Age (years; median, range)	5.0, 1.0-10.0	1.0, 1.0-12.0	4.0, 1.0-10.0	3.0, 1.0-12.0	0.4934	0.7774
Weight (kg; median, range)	20.4, 5.5-31.8	20.9, 7.3-31.6	22.5, 5.0-44.4	20.9, 5.0-44.4	0.3415	0.5703
Sex (female/male)	8/5	4/1	2/4	6/5	*F1=0.4314	*F2=0.3044

NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea; AD = both groups combined (NHD and AHD).
M-W p-value = P-value from Mann-Whitney U test comparing Healthy vs. AD.
K-W p-value = P-value from Kruskal-Wallis test comparing Healthy vs. NHD vs. AHD.
*F1 = P-value from Fisher's exact test comparing proportions for Healthy vs. AD.
*F2 = P-value from Fisher's exact test comparing proportions for Healthy vs. NHD vs. AHD.

454-Pyrosequencing. Bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) was performed targeting the V4-V6 region of the 16S rRNA gene using forward and reverse primers: 530F (5'-GTGCCAGCMGCNGCGG-3') and 1100R (5'-GGGTTNCGNTCGTTG-3'), respectively [70]. Raw sequence data were screened, trimmed, de-noised, filtered, and depleted of chimeras using the QIIME (Quantitative Insights Into Microbial Ecology) open-source pipeline [35]. Operational taxonomic units (OTUs) were assigned based on at least 97% sequence similarity using QIIME.

Quantitative PCR (qPCR) assays. To validate pyrosequencing results and to evaluate bacterial groups that are typically present at very low abundance or not detected in sequencing data, qPCR was used. A panel of seven qPCR assays was performed for specific bacterial groups: *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, *Clostridium perfringens*, *Faecalibacterium* spp., and Veillonellaceae (Table 3). Quantitative PCR was also used to detect the coenzyme A-activated form of butyric acid. A commercially available qPCR thermal cycler (CFX96TM, Bio-Rad Laboratories, CA, USA) was used to execute protocols customized for individual qPCR assays (Table 3 summarizes qPCR assay protocols used). Data was expressed as log amount of DNA per 10 ng of isolated total DNA.

Quantitative PCR reactions were performed using two reaction chemistries. For a subset of assays (Table 3) SYBR-green based reaction mixtures were used, with a total reaction volume of 10 µl. The final mix contained 5 µl SsoFast™ EvaGreen® supermix (Bio-Rad Laboratories, CA, USA), 0.4 µl each of a forward and reverse primer (final

concentration: 400 nM), 2.6 µl of high quality PCR water, and 2 µl of normalized DNA (final concentration: 5 ng/µl). Conditions for PCR were as follows: initial denaturation at 98ºC for 2 min, then 40 cycles with denaturation at 98ºC for 3 sec and annealing (see Table 3 for specific annealing temperatures) for 3 sec. Post-amplification, a melt curve analysis was performed using these conditions: 95ºC for 1 min, 55ºC for 1 min, and increasing incremental steps of 0.5ºC for 80 cycles for 5 sec each. All samples were run in duplicate fashion.

TaqMan® based reaction mixtures were used in a total reaction volume of 10 µl. The final mix contained 5 µl TaqMan® Fast Universal PCR master mix (Life Technologies, NY, USA), 0.4 µl of a forward and reverse primer (final concentration: 400 nM), 2 µl of high quality PCR water, and 2 µl of normalized DNA (final concentration: 5 ng/µl). Conditions for PCR were as follows: initial denaturation at 95ºC for 20 sec then 40 cycles with denaturation at 95°C and annealing (see Table 3 for specific annealing temperatures) for 3 sec. Post-amplification, a melt curve analysis was performed using these conditions: 95ºC for 1 min, 55ºC for 1 min, and increasing incremental steps of 0.5ºC for 80 cycles for 5 sec each. All samples were run in duplicate fashion.

Fatty acid analysis. Concentrations of short-chain fatty acids (SCFA; i.e., acetate, propionate, and butyrate), and branched chain fatty acids (BCFA; i.e., isobutyrate, isovalerate, and valerate) in feces were measured using a stable isotope dilution gas chromatography-mass spectrometry (GC-MS) assay as previously described [71], but with some modifications. Briefly, the fecal samples were weighed and diluted

Table 3. Oligonucleotide primers/probe used for this study.

Primer Sequence (5'-3')	Target	Annealing temperature (°C)	Reference
F-AGCAGTAGGGAATCTTCCA	*Lactobacillus*	58	[72]
R-CACCGCTACACATGGAG			
F-TCGCGTCYGGTGTGAAAG	*Bifidobacterium*	60	[73]
R-CCACATCCAGCRTCCAC			
F-GTTAATACCTTTGCTCATTGA	*Escherichia coli*	55	[73]
R-ACCAGGGTATCTAATCCTGTT			
F-CGCATAACGTTGAAAGATGG	*C. perfringens*	58	[74]
R-CCTTGGTAGGCCGTTACCC			
Probe-TCATCATTCAACCAAAGGAGCAATCC			
F-GAAGGCGGCCTACTGGGCAC	*Faecalibacterium*	60	[75]
R-GTGCAGGCGAGTTGCAGCCT			
F-GGCTHAACCCCRTGAKGGR	Veillonellaceae	61	This study
R-RCCKTGCACCACCTGTYTTC			
F-GCIGAICATTTCACITGGAAYWSITGGCAYATGCCTGC	Butyryl-CoA	53	[76]
R-CTTTGCAATRTCIACRAANGC			
F-TCTGATGTGAAAGGCTGGGGCTTA	*Blautia*	56	[54]
R-GGCTTAGCCACCCGACACCTA			

F = forward primer; R = reverse primer.

1:5 in extraction solution (2 N hydrochloric acid). After homogenization using a multi-tube vortexer for 30 min at room temperature, fecal suspensions were centrifuged for 20 min at 2,100 x g and 4°C. Supernatants were then collected using serum filters (Fisherbrand serum filter system, Fisher Scientific Inc, Pittsburgh, Pa). From each sample, 500 µl of supernatant were mixed with 10 µl of internal standard (200 mM heptadeuterated butyric acid) and extracted using a C18 solid phase extraction column (Sep-Pak C18 1 cc Vac Cartridge, Waters Corporation, Milford, MA). Samples were derivatized using *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) at room temperature for 60 min. A gas chromatographer (Agilent 6890N, Agilent Technologies Inc, Santa Clara, CA) coupled with a mass spectrometer (Agilent 5975C, Agilent Technologies Inc, Santa Clara, CA) was used for chromatographic separation and quantification of the derivatized samples. Separation was achieved using a DB-1ms capillary column (Agilent Technologies Inc, Santa Clara, CA). The GC temperature program was as follows: 40°C held for 0.1 min, increased to 70°C by 5°C/min, 70°C held for 3.5 min, increased to 160°C by 20°C/min, and finally increased to 280°C for 3 min by 35°C/min. The total run time was 20.53 min. The mass spectrometer was operated in electron impact positive-ion mode with selective ion monitoring at mass-to-charge ratios (*M/Z*) of 117 (acetate), 131 (propionate), 145 (butyrate and isobutyrate), 152 (deuterated butyrate; internal standard), and 159 (valerate and isovalerate). Quantification was based on the ratio of the area under the curve of the internal standard for each fatty acid.

For determination of fecal dry weight, a 100 mg sample of feces from each sample was aliquoted into a sterile 1.7 ml serum tube (Microtube, Sarstedt AG & Co, Nümbrecht, Germany). This separate aliquot was weighed and dried at 105°C in an oven (Symphony Gravity Convection Oven, VWR) for 24 hours. The percent of dry weight was calculated and applied to respective concentrations (measured in μmol/g of dry feces for all SCFAs/BCFAs) to calculate total SCFA/BCFA concentrations normalized by dry weight.

Statistical analyses. For sequence analysis, to account for unequal sequencing depth across samples and to avoid exclusion of samples with lower number of sequence reads, the subsequent analysis was performed on a randomly selected subset of 6,900 sequences per sample. Differences in microbial communities between healthy dogs, dogs with NHD, and dogs with AHD were analyzed using the phylogeny-based unweighted UniFrac distance metric, and PCoA plots and rarefaction curves were plotted using QIIME [35]. Rarefaction curves and PCoA plots illustrated alpha (e.g., Chao 1, Shannon Index, and Observed Species) and beta (microbial community distance matrix) diversity. ANOSIM function, or "Analysis of Similarity" from the software package PRIMER 6 (PRIMER-E Ltd., Luton, UK), was used to determine significant differences in the composition of the microbiota between healthy dogs and diseased dogs. To visualize the relative abundance of bacterial families for individual fecal samples, heat maps were generated in NCSS 2007 (NCSS, Kaysville, Utah).

All datasets were tested for normality using the Shapiro-Wilk test (JMP 10, SAS software Inc.). Only bacterial taxa that were present in at least 70% of dogs (either

healthy or diseased) were included in 454-pyrosequencing data analysis. Because most datasets did not meet the assumptions of normal distribution, the differences in the proportions of bacterial taxa (defined as percentage of total sequences), qPCR results (defined as log DNA), functional groups (defined as relative number of KEGG orthologs), and SCFAs and BCFAs (defined as μmol/g of dry feces or percent of total SCFAs) between healthy and disease groups were determined using non-parametric Kruskal-Wallis tests (healthy dogs vs. dogs with NHD vs. dogs with AHD) or a Mann-Whitney U test (healthy dogs vs. dogs with acute diarrhea (AD [dogs with NHD and dogs with AHD combined]). The resulting p-values of the Kruskal-Wallis tests or Mann-Whitney U test were corrected for multiple comparisons using the Benjamini & Hochberg's False Discovery Rate (FDR), and an adjusted $p<0.05$ was considered statistically significant [77]. For those groups (e.g. bacterial groups, SCFAs and BCFAs) that were still significant after p-value adjustment, a Dunn's post-test was used to determine, which animal phenotype were significantly different from the rest. In addition to this, SCFA comparisons were performed using two different approaches, one evaluated total SCFA and total BCFA concentrations normalized by dry weight (defined as μmol/g of dry feces), the other evaluated specific SCFA concentrations (i.e., acetic acid, butyric acid, and propionic acid) as a percent of the total SCFAs.

Linear discriminant analysis effect size (LEfSe) was performed to elucidate statistical and biological significance of the microbiota data obtained from 454-pyrosequencing. LEfSe is freely available online in the Galaxy workflow framework [78,79]. A histogram of the linear discriminant analysis scores was constructed to

express bacterial groups differentially abundant between healthy dogs and dogs with AD. LEfSe scores can be useful in interpreting the degree of difference in the relative abundance between features in the two classes (e.g., healthy dogs and dogs with AD) of analyzed microbial communities [80].

454-pyrosequencing of the 16S rRNA gene is a key tool for studying microbial communities. However, it does not provide direct evidence of the functional capabilities of a bacterial community. For our study, PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to perform ancestral state reconstruction, along with a weighting method, to make predictions of the gene content (with estimates of uncertainty) for all organisms represented in the Greengenes phylogenetic tree of 16S gene sequences [81]. Any functional classification scheme can be used with PICRUSt; in our study, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology classification scheme [82]. The number of KEGG orthologs belonging to functional categories was normalized among levels 1, 2, and 3 in order to evenly represent KEGG orthologs across all groups of dogs. PICRUSt is freely available online in the Galaxy workflow framework and can also be used through QIIME open-source pipeline [78,79].

RESULTS

Sequence analysis. The analysis pipeline yielded 297,315 quality sequences for the 19 samples analyzed (mean ± standard deviation [of all samples] = 9013.7 ± 1203.9). To account for unequal sequencing depth across samples and to avoid excluding samples

with lower number of sequencing reads, the subsequent analysis was performed on a randomly selected subset of 6,900 sequences per sample. Figures 1, 2, and 3 illustrate the rarefaction curves for the Chao1 metric (an estimate of true species richness), observed species (a count of all unique operational taxonomic units (OTUs)), and Shannon Index (describes bacterial diversity by taking into account species richness and evenness). The aforementioned diversity measures are summarized in Table 4. The Shannon Index was significantly decreased in dogs with AD (mean ± standard deviation (SD): 4.0 ± 0.6) relative to healthy dogs (mean ± SD: 4.8 ± 0.4; p=0.0022). The Shannon Index was significantly decreased in dogs with NHD (mean ± SD: 4.0 ± 0.2) compared to healthy dogs (mean ± SD: 4.8 ± 0.4; $p<0.05$). No significant differences were seen for observed species and Chao 1 across the dog groups.

The percent of sequences (phylum and genus level) observed in all dogs are illustrated in Figure 4 and 5, respectively. Bacterial groups included in the statistical analysis are described in Table 5. All bacterial groups (e.g., including bacterial groups present with low abundance) regardless of being included in statistical analysis are described in Table 6. Sequences belonging to the phylum Bacteroidetes were significantly decreased in dogs with AD compared to healthy dogs (p=0.0280). Sequences belonging to the genus *Faecalibacterium* and an unclassified genus within the family Ruminococcaceae were both significantly decreased in dogs with AD compared to healthy dogs (p=0.0319 and 0.0368, respectively). Sequences belonging to the genus *Clostridium* were significantly increased in dogs with AD compared to healthy dogs (p=0.0476).

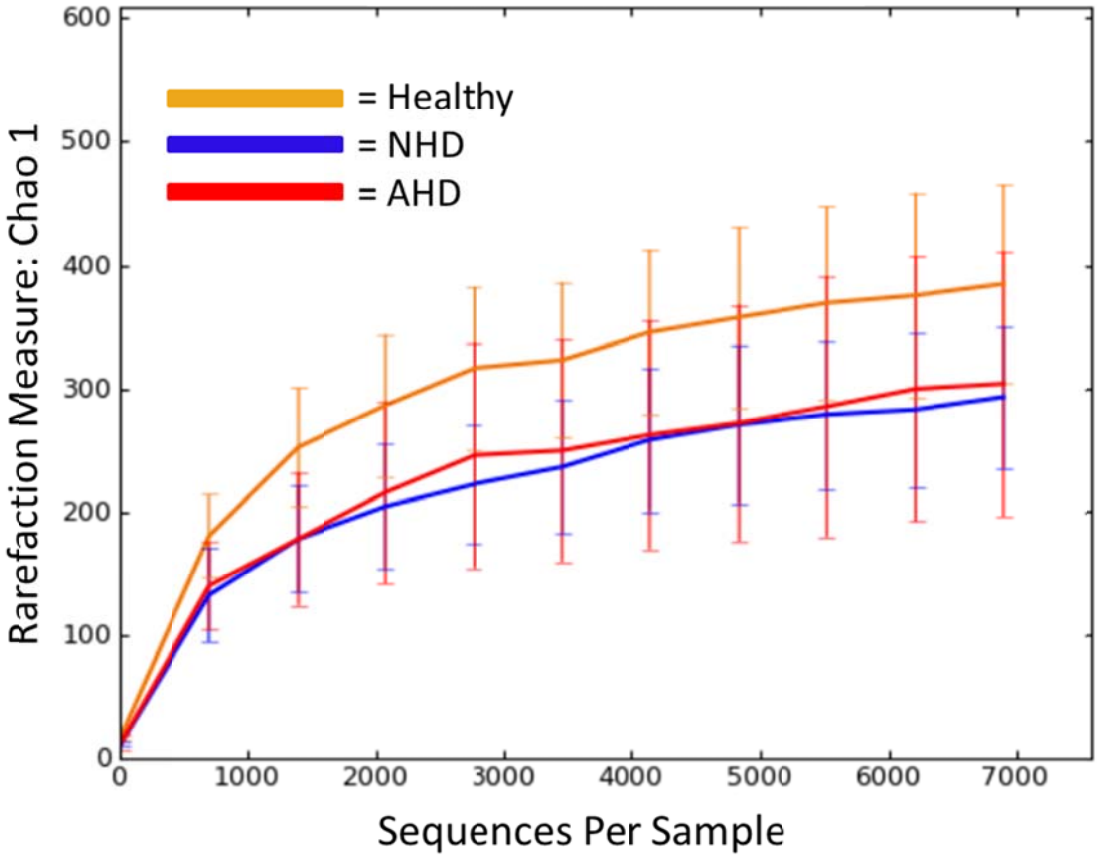

Figure 1. **Rarefaction curves for the Chao 1 metric based on 16S rRNA gene sequences obtained from canine fecal samples.** Lines represent the mean and error bars represent standard deviations. The analysis was performed on a randomly selected subset of 6,900 sequences per sample. Healthy = healthy dogs; NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea.

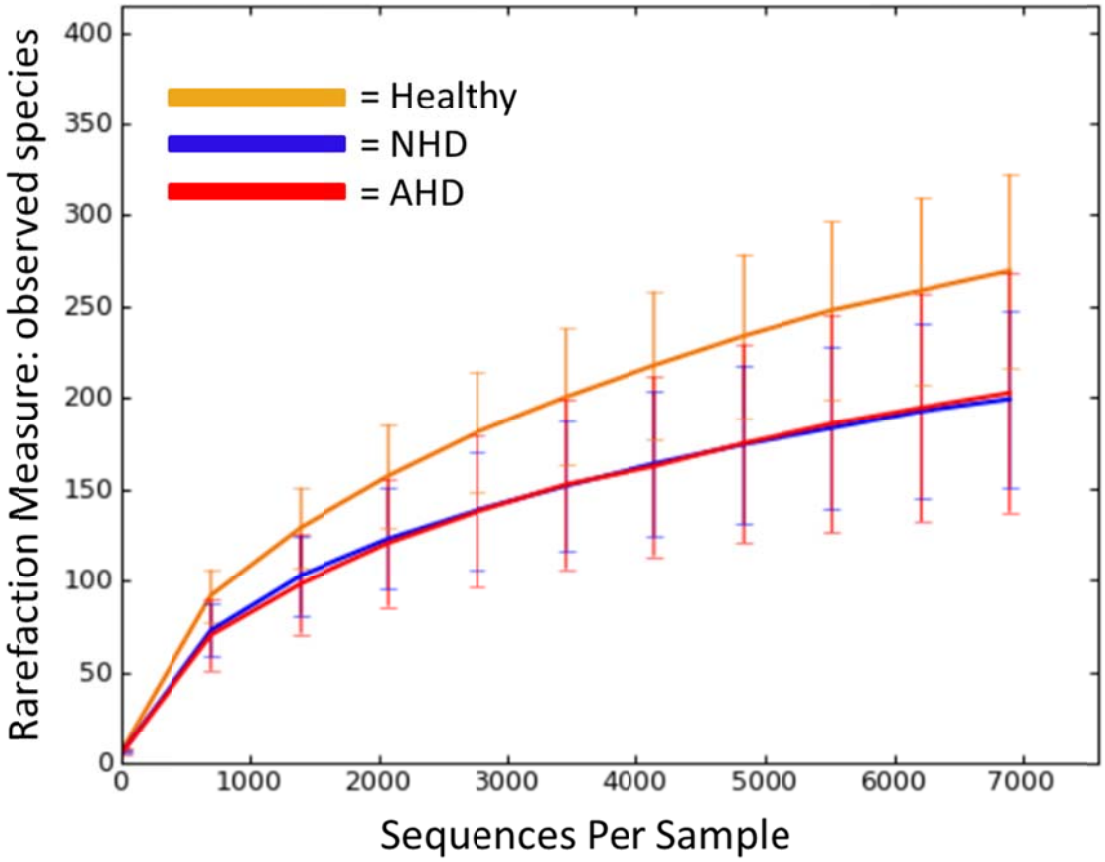

Figure 2. **Rarefaction curves for observed species based on 16S rRNA gene sequences obtained from canine fecal samples.** Lines represent the mean and error bars represent standard deviations. The analysis was performed on a randomly selected subset of 6,900 sequences per sample. Healthy = healthy dogs; NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea.

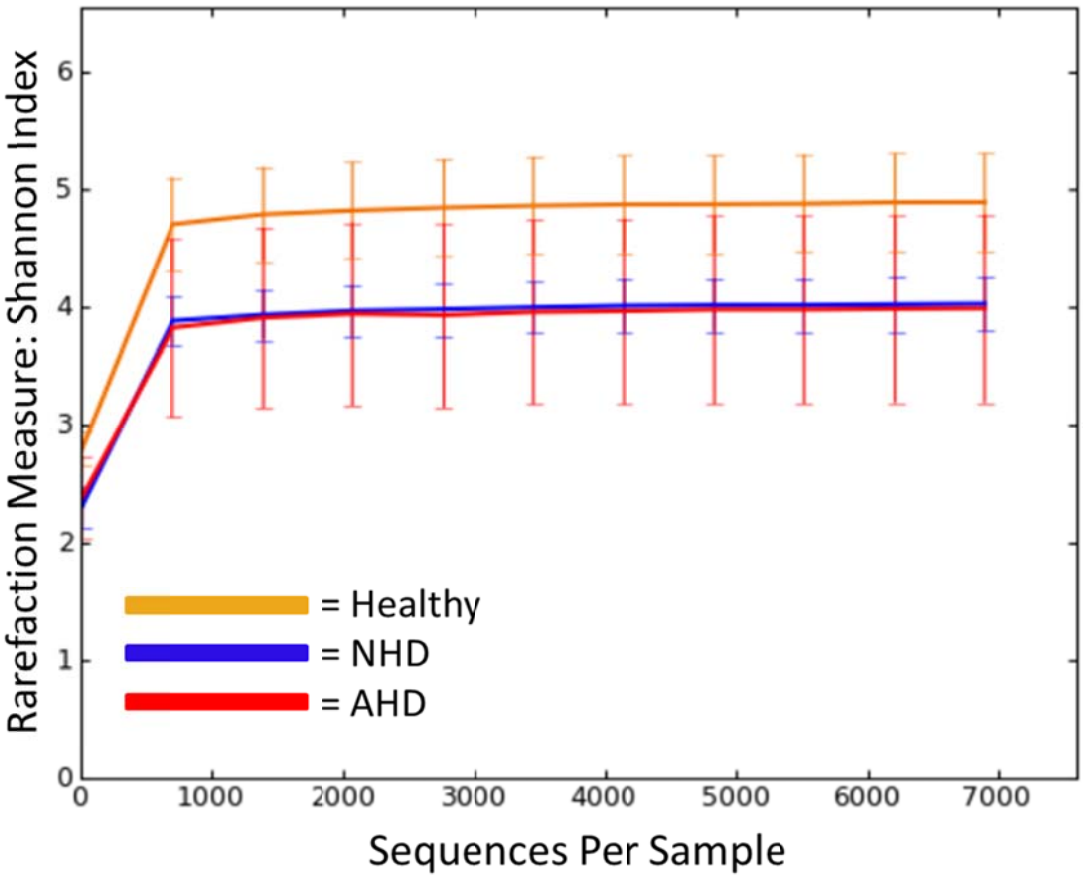

Figure 3. Rarefaction curves for Shannon Index based on 16S rRNA gene sequences obtained from canine fecal samples. Lines represent the mean and error bars represent standard deviations. The analysis was performed on a randomly selected subset of 6,900 sequences per sample. Healthy = healthy dogs; NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea.

Table 4. Summary of alpha diversity measures.

	Mean ± SD					
	Healthy	**AD**	**NHD**	**AHD**	**M-W p-value**	**K-W p-value**
Observed Species	268.1±52.8	201.3±56.7	200.3±48.5	204.6±35.8	0.0519	0.1465
Shannon Index	4.8[a]±0.4	4.0±0.6	4.0[b]±0.2	3.9[a,b]±0.8	**0.0022**	**0.0085**
Chao1	386.1±80.6	303.6±87.0	291.2±57.7	305.8±109.7	0.3637	0.3300

NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea; AD = both groups combined (NHD and AHD).
M-W p-value = P-value from Mann-Whitney U test comparing healthy dogs and dogs with AD.
K-W p-value = P-value from Kruskal-Wallis test comparing healthy dogs, dogs with NHD, and dogs with AHD.
Means not sharing a common superscript are significantly different ($p<0.05$; Dunn's multiple comparisons test).

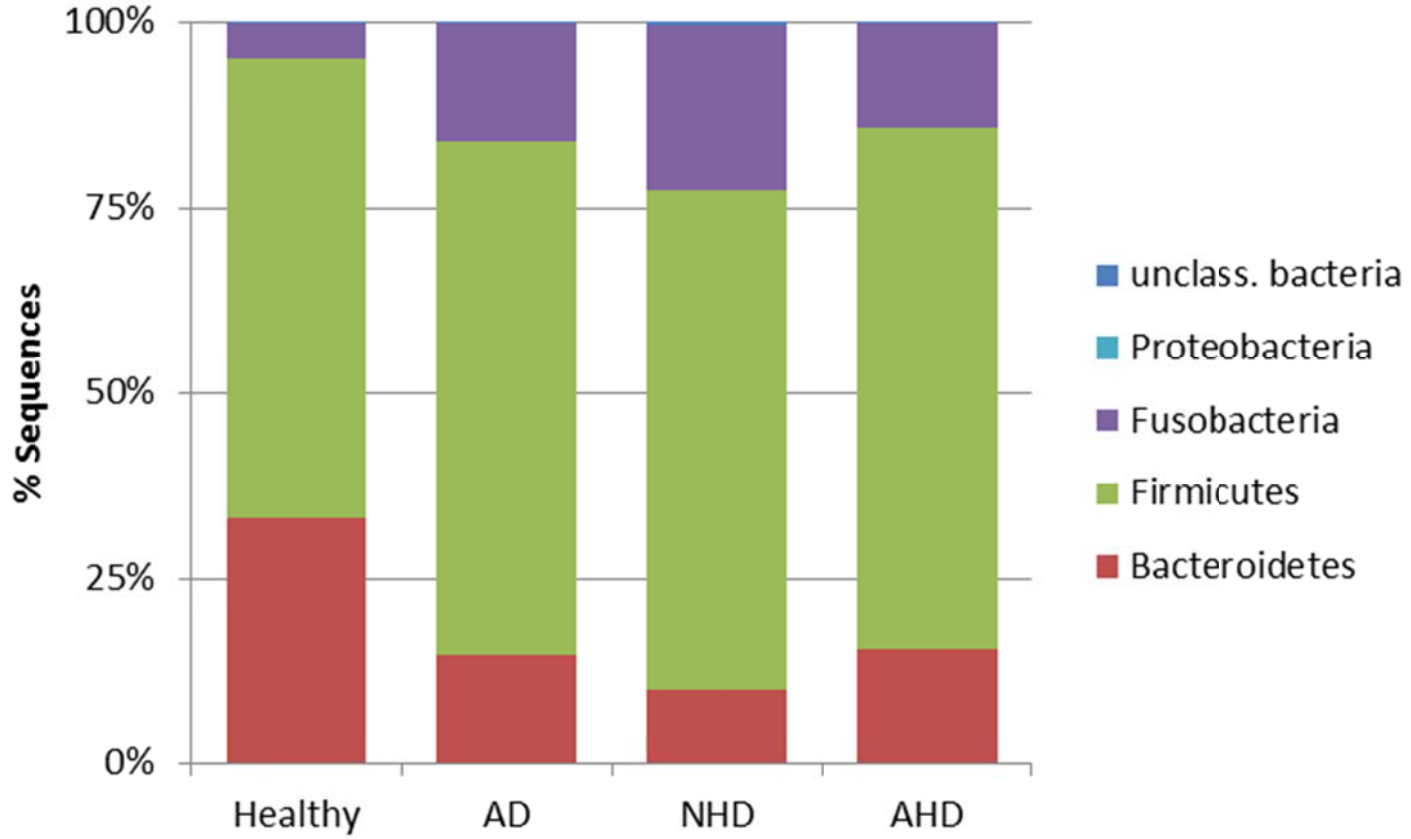

Figure 4. **Percent of sequences for observed bacterial phyla.** Healthy = healthy dogs; NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea; AD = both groups combined (NHD and AHD). The abbreviation "unclass." denotes an unclassified phylum within the domain bacteria.

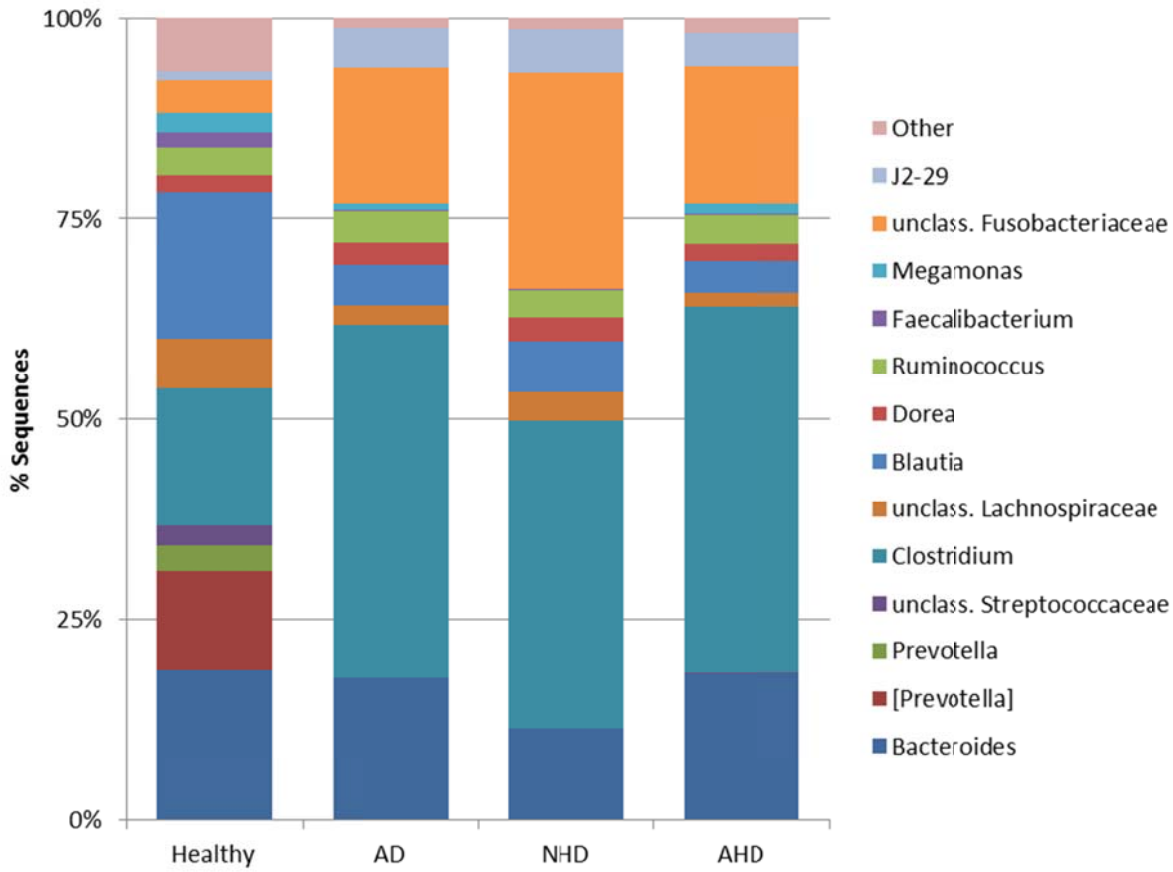

Figure 5. Percent of sequences for observed bacterial genera. Healthy = Healthy dogs; NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea; AD = both groups combined (NHD and AHD). The abbreviation "unclass." denotes an unclassified genus within the respective bacterial families. Brackets "[]" denote a proposed taxonomy that has not yet been verified.

Table 5. Relative percentages of bacteria included in statistical analysis at various phylogenetic levels based on pyrosequencing.

	median(min-max) *in percent					
Taxa	**Healthy**	**AD**	**NHD**	**AHD**	**M-W p-value**	**K-W p-value**
Phylum						
Bacteroidetes	32.6(12.9-48.4)	15.3(0.0-27.9)	10.5(0.0-16.1)	15.5(0.1-27.9)	0.0280	0.0805
Firmicutes	60.9(41.3-86.6)	72.2(32.7-99.9)	72.2(32.7-99.9)	71.3(47.5-99.8)	0.7213	0.9918
Fusobacteria	4.5(0.1-12.7)	16.5(0.1-49.5)	23.5(0.1-49.5)	14.2(0.1-24.2)	0.0793	0.1915
Proteobacteria	0.1(0.0-0.3)	0.1(0.0-1.2)	0.1(0.0-1.2)	0.1(0.0-0.2)	0.9596	0.6914
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)	0.9008	0.8398
Class						
Bacilli	2.8(0.1-31.8)	4.6(0.0-77.5)	2.0(0.0-43.3)	10.0(0.0-77.5)	0.9639	0.9360
Bacteroidia	32.6(12.9-48.4)	15.3(0.0-27.9)	10.5(0.0-16.1)	15.5(0.1-27.9)	0.0504	0.1449
Clostridia	54.0(26.2-66.1)	47.2(21.8-74.5)	50.2(30.6-64.8)	44.7(21.8-74.5)	1.0653	0.9097
Epsilonproteobacteria	0.0(0.0-0.1)	0.0(0.0-0.4)	0.0(0.0-0.4)	0.0(0.0-0.1)	0.9202	1.0712
Erysipelotrichi	4.2(1.5-19.0)	0.4(0.0-6.7)	0.4(0.0-6.7)	0.6(0.2-3.3)	0.0531	0.1413
Fusobacteria	4.5(0.1-12.7)	16.5(0.1-49.5)	23.5(0.1-49.5)	14.2(0.1-24.2)	0.0951	0.2298
Gammaproteobacteria	0.0(0.0-0.3)	0.0(0.0-1.1)	0.0(0.0-1.1)	0.0(0.0-0.1)	1.1057	0.9079
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)	1.0134	1.2092
unclass. Firmicutes	0.2(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.2)	0.9095	0.9225
Order						
Bacteroidales	32.6(12.9-48.4)	15.3(0.0-27.9)	10.5(0.0-16.1)	15.5(0.1-27.9)	0.0728	0.2093
Campylobacterales	0.0(0.0-0.1)	0.0(0.0-0.4)	0.0(0.0-0.4)	0.0(0.0-0.1)	0.9202	0.8439
Clostridiales	52.3(25.7-65.7)	46.7(21.7-73.7)	50.1(29.8-63.7)	44.3(21.7-73.7)	0.9131	0.9433
Coriobacteriales	0.3(0.1-1.8)	0.1(0.0-0.6)	0.0(0.0-0.6)	0.1(0.0-0.4)	0.1464	0.3708
Enterobacteriales	0.0(0.0-0.1)	0.0(0.0-1.1)	0.0(0.0-1.1)	0.0(0.0-0.0)	0.9685	0.6302
Erysipelotrichales	4.2(1.5-19.0)	0.4(0.0-6.7)	0.4(0.0-6.7)	0.6(0.2-3.3)	0.0767	0.2041
Fusobacteriales	4.5(0.1-12.7)	16.5(0.1-49.5)	23.5(0.1-49.5)	14.2(0.1-24.2)	0.1030	0.2490

Table 5. Continued.

	median(min-max) *in percent					
Taxa	**Healthy**	**AD**	**NHD**	**AHD**	**M-W p-value**	**K-W p-value**
Order						
Lactobacillales	2.2(0.1-31.7)	4.6(0.0-47.4)	0.6(0.0-43.3)	10.0(0.0-47.4)	1.0075	0.8565
Turicibacterales	0.0(0.0-2.2)	0.0(0.0-61.1)	0.0(0.0-1.9)	0.0(0.0-61.1)	0.2745	0.5362
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)	0.9759	0.8733
unclass. Clostridia_1	0.4(0.1-0.9)	0.1(0.0-0.5)	0.1(0.0-0.5)	0.2(0.0-0.3)	0.0793	0.2327
unclass. Clostridia_2	0.0(0.0-1.1)	0.0(0.0-0.7)	0.1(0.0-0.7)	0.0(0.0-0.0)	0.9801	0.4489
unclass. Firmicutes	0.2(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.2)	0.7507	0.6663
Family						
Bacteroidaceae	14.2(0.6-32.7)	12.6(0.0-18.9)	9.2(0.0-16.1)	12.7(0.0-18.9)	0.4133	0.1957
Clostridiaceae	13.2(4.7-16.0)	33.0(1.0-53.9)	33.0(1.0-43.8)	31.8(17.4-53.9)	0.0884	0.2490
Coprobacillaceae	2.6(0.6-13.7)	0.2(0.0-5.5)	0.2(0.0-5.5)	0.3(0.0-1.6)	0.0910	0.1995
Coriobacteriaceae	0.3(0.1-1.8)	0.1(0.0-0.6)	0.0(0.0-0.6)	0.1(0.0-0.4)	0.1626	0.1622
Enterobacteriaceae	0.0(0.0-0.1)	0.0(0.0-1.1)	0.0(0.0-1.1)	0.0(0.0-0.0)	0.8422	0.5521
Enterococcaceae	0.0(0.0-0.2)	0.0(0.0-10.2)	0.0(0.0-0.1)	0.0(0.0-10.2)	0.8508	0.4170
Erysipelotrichaceae	1.9(0.4-5.4)	0.4(0.0-5.8)	0.4(0.0-5.8)	0.4(0.0-1.6)	0.0681	0.1326
Fusobacteriaceae	4.5(0.1-12.7)	16.5(0.1-49.5)	23.5(0.1-49.5)	14.2(0.1-24.2)	0.1177	0.6987
Helicobacteraceae	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.8691	0.4144
Lachnospiraceae	25.8(16.8-39.7)	8.7(1.6-48.8)	8.7(5.6-48.8)	8.4(1.6-32.0)	0.1401	0.4415
Paraprevotellaceae	2.5(0.0-11.6)	0.0(0.0-7.8)	0.0(0.0-1.3)	1.3(0.0-7.8)	0.2958	0.4590
Peptococcaceae	0.2(0.0-1.3)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.0(0.0-0.1)	0.3332	0.5125
Peptostreptococcaceae	0.3(0.1-5.8)	0.1(0.0-4.8)	0.1(0.0-3.5)	0.1(0.0-4.8)	0.3809	0.1898
Prevotellaceae	9.5(0.0-25.1)	0.0(0.0-1.7)	0.0(0.0-0.0)	0.0(0.0-1.7)	0.0832	0.4649
Ruminococcaceae	1.8(0.2-5.7)	0.3(0.0-1.1)	0.7(0.0-1.1)	0.3(0.1-1.1)	0.0797	0.2288
Streptococcaceae	2.2(0.1-11.6)	0.0(0.0-47.4)	0.0(0.0-43.3)	0.1(0.0-47.4)	0.3330	0.4402

Table 5. Continued.

	median(min-max) *in percent					
Taxa	**Healthy**	**AD**	**NHD**	**AHD**	**M-W p-value**	**K-W p-value**
Family						
Succinivibrionaceae	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.2701	0.5330
Turicibacteraceae	0.0(0.0-2.2)	0.0(0.0-61.1)	0.0(0.0-1.9)	0.0(0.0-61.1)	0.2995	0.1803
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)	0.9008	0.5402
unclass. Bacteroidales	0.0(0.0-0.2)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)	0.8367	0.5300
unclass. Clostridia_1	0.4(0.1-0.9)	0.1(0.0-0.5)	0.1(0.0-0.5)	0.2(0.0-0.3)	0.0793	0.3235
unclass. Clostridia_2	0.0(0.0-1.1)	0.0(0.0-0.7)	0.1(0.0-0.7)	0.0(0.0-0.0)	0.8019	0.4349
unclass. Clostridiales	0.1(0.0-0.3)	0.2(0.0-0.3)	0.2(0.1-0.3)	0.2(0.0-0.3)	0.3181	17.4668
unclass. Firmicutes	0.2(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.2)	0.5255	0.3371
unclass. Lactobacillales	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.3089	0.8126
Veillonellaceae	3.0(0.1-21.6)	0.6(0.0-3.9)	0.1(0.0-2.0)	0.9(0.4-3.9)	0.3215	0.2096
Genus						
Allobaculum	0.6(0.0-1.9)	0.1(0.0-5.7)	0.1(0.0-5.7)	0.1(0.0-0.6)	0.4284	0.6893
Bacteroides	14.2(0.6-32.7)	12.5(0.0-18.9)	9.2(0.0-16.1)	12.6(0.0-18.9)	0.4530	0.6348
Blautia	14.0(8.9-25.2)	3.6(0.2-19.8)	5.1(0.9-19.8)	2.7(0.2-17.0)	0.0977	0.2216
Catenibacterium	0.3(0.0-13.2)	0.1(0.0-1.6)	0.0(0.0-0.9)	0.1(0.0-1.6)	0.2893	0.4440
Clostridium	13.2(4.7-16.0)	31.2(0.3-53.8)	31.2(0.3-43.8)	31.8(17.2-53.8)	0.0476	0.1428
Collinsella	0.3(0.1-1.7)	0.0(0.0-0.4)	0.0(0.0-0.4)	0.1(0.0-0.3)	0.1058	0.3058
Dorea	1.5(0.3-12.6)	2.0(0.2-9.8)	2.4(0.2-9.8)	1.6(0.2-6.3)	0.8286	0.9129
Enterococcus	0.0(0.0-0.2)	0.0(0.0-9.2)	0.0(0.0-0.1)	0.0(0.0-9.2)	0.8810	0.8815
Escherichia	0.0(0.0-0.1)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.0(0.0-0.0)	0.9574	0.7592
Eubacterium	1.0(0.4-3.8)	0.1(0.0-1.0)	0.1(0.0-0.4)	0.2(0.0-1.0)	0.0504	0.1764
Faecalibacterium	1.5(0.1-5.4)	0.1(0.0-1.1)	0.0(0.0-1.1)	0.1(0.0-0.9)	0.0319	0.1067
Helicobacter	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.8866	0.8981

Table 5. Continued.

	median(min-max) *in percent					
Taxa	**Healthy**	**AD**	**NHD**	**AHD**	**M-W p-value**	**K-W p-value**
Genus						
J2-29	0.8(0.0-2.8)	3.5(0.0-8.2)	4.3(0.0-7.1)	2.9(0.0-8.2)	0.2570	0.4603
Megamonas	1.9(0.1-20.5)	0.6(0.0-1.9)	0.1(0.0-1.9)	0.9(0.2-1.8)	0.4043	0.3563
Peptococcus	0.2(0.0-1.3)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.0(0.0-0.1)	0.3683	0.5052
Phascolarctobacterium	0.0(0.0-1.3)	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.2)	0.1953	0.4377
Prevotella	9.5(0.0-25.1)	0.0(0.0-1.7)	0.0(0.0-0.0)	0.0(0.0-1.7)	0.0896	0.1872
[Prevotella]	2.5(0.0-11.6)	0.0(0.0-7.8)	0.0(0.0-0.1)	0.0(0.0-7.8)	0.2358	0.3906
[Ruminococcus]	2.7(0.7-10.6)	2.8(0.1-15.6)	2.8(0.3-15.6)	2.6(0.1-7.0)	0.8770	0.9044
Ruminococcus	0.1(0.0-0.2)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.1(0.0-0.3)	0.7934	0.8811
Slackia	0.0(0.0-0.1)	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.2)	0.7038	0.8714
Streptococcus	0.3(0.0-1.3)	0.0(0.0-9.7)	0.0(0.0-2.3)	0.0(0.0-9.7)	0.3614	0.5124
Turicibacter	0.0(0.0-2.2)	0.0(0.0-61.1)	0.0(0.0-1.9)	0.0(0.0-61.1)	0.2956	0.5272
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)	0.9228	0.9041
unclass. Bacteroidales	0.0(0.0-0.2)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)	0.8348	0.6496
unclass. Clostridia_1	0.4(0.1-0.9)	0.1(0.0-0.5)	0.1(0.0-0.5)	0.2(0.0-0.3)	0.1098	0.2506
unclass. Clostridia_2	0.0(0.0-1.1)	0.0(0.0-0.7)	0.1(0.0-0.7)	0.0(0.0-0.0)	0.8142	0.4580
unclass. Clostridiaceae	0.0(0.0-2.7)	0.0(0.0-1.8)	0.2(0.0-1.8)	0.0(0.0-0.2)	0.8022	0.7486
unclass. Clostridiales	0.1(0.0-0.3)	0.2(0.0-0.3)	0.2(0.1-0.3)	0.2(0.0-0.3)	0.3854	0.5120
unclass. Coprobacillaceae	0.7(0.0-2.6)	0.0(0.0-5.4)	0.0(0.0-5.4)	0.0(0.0-0.3)	0.1159	0.3293
unclass. Firmicutes	0.2(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.2)	0.5854	0.6150
unclass. Fusobacteriaceae_1	0.0(0.0-0.3)	0.0(0.0-0.2)	0.2(0.0-0.2)	0.0(0.0-0.1)	0.9080	0.4429
unclass. Fusobacteriaceae_2	3.2(0.1-11.8)	12.0(0.0-44.0)	21.9(0.0-44.0)	11.9(0.1-20.6)	0.2534	0.4076
unclass. Lachnospiraceae_1	4.7(3.2-6.7)	1.6(0.2-3.6)	2.9(0.9-3.6)	1.1(0.2-2.9)	0.0840	0.1470
unclass. Lachnospiraceae_2	0.4(0.0-0.6)	0.1(0.0-0.3)	0.1(0.1-0.2)	0.0(0.0-0.3)	0.0905	0.2030

Table 5. Continued.

	median(min-max) *in percent					
Taxa	**Healthy**	**AD**	**NHD**	**AHD**	**M-W p-value**	**K-W p-value**
Genus						
unclass. Lactobacillales	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.3629	0.4180
unclass. Peptostreptococcaceae_1	0.0(0.0-0.0)	0.0(0.0-0.3)	0.0(0.0-0.0)	0.0(0.0-0.3)	0.7815	0.7432
unclass. Peptostreptococcaceae_2	0.3(0.1-5.7)	0.1(0.0-4.5)	0.1(0.0-3.5)	0.1(0.0-4.5)	0.4253	0.6896
unclass. Ruminococcaceae_1	0.1(0.0-0.3)	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.1)	0.0368	0.1292
unclass. Ruminococcaceae_2	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.1)	0.1900	0.3964
unclass. Streptococcaceae	1.8(0.0-10.8)	0.0(0.0-41.0)	0.0(0.0-41.0)	0.0(0.0-37.7)	0.3786	0.6210
unclass. Veilonellaceae	0.1(0.0-1.1)	0.0(0.0-0.4)	0.0(0.0-0.2)	0.0(0.0-0.4)	0.4113	0.6166

NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea; AD = both groups combined (NHD and AHD).
M-W p-value = P-value for Mann-Whitney U test comparing healthy dogs vs. AD adjusted based on the Benjamini and Hochberg False Discovery Rate.
K-W p-value = P-value for Kruskal-Wallis test comparing healthy dogs vs. NHD vs. AHD adjusted based on the Benjamini and Hochberg False Discovery Rate.
The abbreviation “unclass.” denotes an unclassified taxonomy within respective taxonomic group. Brackets “[]” denote a proposed taxonomy that has not yet been verified.

Table 6. Relative percentages of all bacterial groups at the various phylogenetic levels based on 454-pyrosequencing.

	median(min-max) *in percent			
Taxa	**Healthy**	**AD**	**NHD**	**AHD**
Phylum				
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)
Actinobacteria	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
Bacteroidetes	32.6(12.9-48.4)	15.3(0.0-27.9)	10.5(0.0-16.1)	15.5(0.1-27.9)
Firmicutes	60.9(41.3-86.6)	72.2(32.7-99.9)	72.2(32.7-99.9)	71.3(47.5-99.8)
Fusobacteria	4.5(0.1-12.7)	16.5(0.1-49.5)	23.5(0.1-49.5)	14.2(0.1-24.2)
Proteobacteria	0.1(0.0-0.3)	0.1(0.0-1.2)	0.1(0.0-1.2)	0.1(0.0-0.2)
Tenericutes	0.0(0.0-0.1)	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.2)
Class				
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)
Actinobacteria	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
Bacteroidia	32.6(12.9-48.4)	15.3(0.0-27.9)	10.5(0.0-16.1)	15.5(0.1-27.9)
unclass. Firmicutes	0.2(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.2)
Bacilli	2.8(0.1-31.8)	4.6(0.0-77.5)	2.0(0.0-43.3)	10.0(0.0-77.5)
Clostridia	54.0(26.2-66.1)	47.2(21.8-74.5)	50.2(30.6-64.8)	44.7(21.8-74.5)
Erysipelotrichi	4.2(1.5-19.0)	0.4(0.0-6.7)	0.4(0.0-6.7)	0.6(0.2-3.3)
Fusobacteria	4.5(0.1-12.7)	16.5(0.1-49.5)	23.5(0.1-49.5)	14.2(0.1-24.2)
Betaproteobacteria	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)
Epsilonproteobacteria	0.0(0.0-0.1)	0.0(0.0-0.4)	0.0(0.0-0.4)	0.0(0.0-0.1)
Gammaproteobacteria	0.0(0.0-0.3)	0.0(0.0-1.1)	0.0(0.0-1.1)	0.0(0.0-0.1)
Mollicutes	0.0(0.0-0.1)	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.2)
Order				
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)
Actinomycetales	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Bifidobacteriales	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)

Table 6. Continued.

	median(min-max) *in percent			
Taxa	**Healthy**	**AD**	**NHD**	**AHD**
Order				
Bacteroidales	32.6(12.9-48.4)	15.3(0.0-27.9)	10.5(0.0-16.1)	15.5(0.1-27.9)
unclass. Firmicutes	0.2(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.2)
unclass. Bacilli	0.0(0.0-0.0)	0.0(0.0-0.9)	0.0(0.0-0.0)	0.0(0.0-0.9)
Lactobacillales	2.2(0.1-31.7)	4.6(0.0-47.4)	0.6(0.0-43.3)	10.0(0.0-47.4)
Turicibacterales	0.0(0.0-2.2)	0.0(0.0-61.1)	0.0(0.0-1.9)	0.0(0.0-61.1)
unclass. Clostridia_1	0.4(0.1-0.9)	0.1(0.0-0.5)	0.1(0.0-0.5)	0.2(0.0-0.3)
unclass. Clostridia_2	0.0(0.0-1.1)	0.0(0.0-0.7)	0.1(0.0-0.7)	0.0(0.0-0.0)
Clostridiales	52.3(25.7-65.7)	46.7(21.7-73.7)	50.1(29.8-63.7)	44.3(21.7-73.7)
Coriobacteriales	0.3(0.1-1.8)	0.1(0.0-0.6)	0.0(0.0-0.6)	0.1(0.0-0.4)
Erysipelotrichales	4.2(1.5-19.0)	0.4(0.0-6.7)	0.4(0.0-6.7)	0.6(0.2-3.3)
Fusobacteriales	4.5(0.1-12.7)	16.5(0.1-49.5)	23.5(0.1-49.5)	14.2(0.1-24.2)
Burkholderiales	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)
Campylobacterales	0.0(0.0-0.1)	0.0(0.0-0.4)	0.0(0.0-0.4)	0.0(0.0-0.1)
Aeromonadales	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Enterobacteriales	0.0(0.0-0.1)	0.0(0.0-1.1)	0.0(0.0-1.1)	0.0(0.0-0.0)
Pasteurellales	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
Pseudomonadales	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. Mollicutes	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Anaeroplasmatales	0.0(0.0-0.0)	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.2)
RF39	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)
Microbacteriaceae	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Nocardioidaceae	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Bifidobacteriaceae	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)

Table 6. Continued.

	median(min-max) *in percent			
Taxa	**Healthy**	**AD**	**NHD**	**AHD**
Family				
unclass. Bacteroidales	0.0(0.0-0.2)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
Bacteroidaceae	14.2(0.6-32.7)	12.6(0.0-18.9)	9.2(0.0-16.1)	12.7(0.0-18.9)
Porphyromonadaceae	0.0(0.0-0.8)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Prevotellaceae	9.5(0.0-25.1)	0.0(0.0-1.7)	0.0(0.0-0.0)	0.0(0.0-1.7)
S24-7	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
[Odoribacteraceae]	0.0(0.0-0.3)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
[Paraprevotellaceae]	2.5(0.0-11.6)	0.0(0.0-7.8)	0.0(0.0-1.3)	1.3(0.0-7.8)
unclass. Firmicutes	0.2(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.2)
unclass. Bacilli	0.0(0.0-0.0)	0.0(0.0-0.9)	0.0(0.0-0.0)	0.0(0.0-0.9)
unclass. Lactobacillales	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)
Enterococcaceae	0.0(0.0-0.2)	0.0(0.0-10.2)	0.0(0.0-0.1)	0.0(0.0-10.2)
Lactobacillaceae	0.0(0.0-25.9)	0.0(0.0-26.7)	0.0(0.0-26.7)	0.0(0.0-15.2)
Leuconostocaceae	0.0(0.0-0.0)	0.0(0.0-5.4)	0.0(0.0-0.0)	0.0(0.0-5.4)
Streptococcaceae	2.2(0.1-11.6)	0.0(0.0-47.4)	0.0(0.0-43.3)	0.1(0.0-47.4)
Turicibacteraceae	0.0(0.0-2.2)	0.0(0.0-61.1)	0.0(0.0-1.9)	0.0(0.0-61.1)
unclass. Clostridia_1	0.4(0.1-0.9)	0.1(0.0-0.5)	0.1(0.0-0.5)	0.2(0.0-0.3)
unclass. Clostridia_2	0.0(0.0-1.1)	0.0(0.0-0.7)	0.1(0.0-0.7)	0.0(0.0-0.0)
unclass. Clostridiales	0.1(0.0-0.3)	0.2(0.0-0.3)	0.2(0.1-0.3)	0.2(0.0-0.3)
Clostridiaceae	13.2(4.7-16.0)	33.0(1.0-53.9)	33.0(1.0-43.8)	31.8(17.4-53.9)
Lachnospiraceae	25.8(16.8-39.7)	8.7(1.6-48.8)	8.7(5.6-48.8)	8.4(1.6-32.0)
Peptococcaceae	0.2(0.0-1.3)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.0(0.0-0.1)
Peptostreptococcaceae	0.3(0.1-5.8)	0.1(0.0-4.8)	0.1(0.0-3.5)	0.1(0.0-4.8)
Ruminococcaceae	1.8(0.2-5.7)	0.3(0.0-1.1)	0.7(0.0-1.1)	0.3(0.1-1.1)
Veillonellaceae	3.0(0.1-21.6)	0.6(0.0-3.9)	0.1(0.0-2.0)	0.9(0.4-3.9)

Table 6. Continued.

	median(min-max) *in percent			
Taxa	**Healthy**	**AD**	**NHD**	**AHD**
Family				
Coriobacteriaceae	0.3(0.1-1.8)	0.1(0.0-0.6)	0.0(0.0-0.6)	0.1(0.0-0.4)
unclass. Erysipelotrichales_1	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. Erysipelotrichales_2	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Erysipelotrichaceae	1.9(0.4-5.4)	0.4(0.0-5.8)	0.4(0.0-5.8)	0.4(0.0-1.6)
[Coprobacillaceae]	2.6(0.6-13.7)	0.2(0.0-5.5)	0.2(0.0-5.5)	0.3(0.0-1.6)
Fusobacteriaceae	4.5(0.1-12.7)	16.5(0.1-49.5)	23.5(0.1-49.5)	14.2(0.1-24.2)
Alcaligenaceae	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)
Campylobacteraceae	0.0(0.0-0.0)	0.0(0.0-0.3)	0.0(0.0-0.3)	0.0(0.0-0.0)
Helicobacteraceae	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)
Succinivibrionaceae	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Enterobacteriaceae	0.0(0.0-0.1)	0.0(0.0-1.1)	0.0(0.0-1.1)	0.0(0.0-0.0)
Pasteurellaceae	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
Pseudomonadaceae	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. Mollicutes	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Anaeroplasmataceae	0.0(0.0-0.0)	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.2)
unclass. RF39	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Genus				
unclass. bacteria	0.2(0.0-0.8)	0.3(0.0-0.4)	0.4(0.0-0.4)	0.3(0.0-0.3)
Leucobacter	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. Nocardioidaceae	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Bifidobacterium	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
unclass. Bacteroidales	0.0(0.0-0.2)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
unclass. Bacteroidaceae	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
Bacteroides	14.2(0.6-32.7)	12.5(0.0-18.9)	9.2(0.0-16.1)	12.6(0.0-18.9)

Table 6. Continued.

	median(min-max) *in percent			
Taxa	**Healthy**	**AD**	**NHD**	**AHD**
Genus				
Parabacteroides	0.0(0.0-0.8)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Prevotella	9.5(0.0-25.1)	0.0(0.0-1.7)	0.0(0.0-0.0)	0.0(0.0-1.7)
unclass. S24-7	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)
Odoribacter	0.0(0.0-0.3)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. [Paraprevotellaceae]	0.0(0.0-0.1)	0.0(0.0-2.7)	0.0(0.0-1.2)	0.0(0.0-2.7)
[Prevotella]	2.5(0.0-11.6)	0.0(0.0-7.8)	0.0(0.0-0.1)	0.0(0.0-7.8)
unclass. Firmicutes	0.2(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.4)	0.1(0.0-0.2)
unclass. Bacilli	0.0(0.0-0.0)	0.0(0.0-0.9)	0.0(0.0-0.0)	0.0(0.0-0.9)
unclass. Lactobacillales	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)
Enterococcaceae	0.0(0.0-0.0)	0.0(0.0-0.9)	0.0(0.0-0.0)	0.0(0.0-0.9)
Enterococcus	0.0(0.0-0.2)	0.0(0.0-9.2)	0.0(0.0-0.1)	0.0(0.0-9.2)
unclass. Lactobacillaceae_1	0.0(0.0-0.1)	0.0(0.0-25.7)	0.0(0.0-25.7)	0.0(0.0-14.9)
unclass. Lactobacillaceae_2	0.0(0.0-0.0)	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.0)
Lactobacillus	0.0(0.0-25.9)	0.0(0.0-0.8)	0.0(0.0-0.8)	0.0(0.0-0.2)
unclass. Leuconostocaceae	0.0(0.0-0.0)	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.2)
Weissella	0.0(0.0-0.0)	0.0(0.0-5.2)	0.0(0.0-0.0)	0.0(0.0-5.2)
unclass. Streptococcaceae_1	1.8(0.0-10.8)	0.0(0.0-41.0)	0.0(0.0-41.0)	0.0(0.0-37.7)
unclass. Streptococcaceae_2	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Lactococcus	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Streptococcus	0.3(0.0-1.3)	0.0(0.0-9.7)	0.0(0.0-2.3)	0.0(0.0-9.7)
Turicibacter	0.0(0.0-2.2)	0.0(0.0-61.1)	0.0(0.0-1.9)	0.0(0.0-61.1)
unclass. Clostridia_1	0.4(0.1-0.9)	0.1(0.0-0.5)	0.1(0.0-0.5)	0.2(0.0-0.3)
unclass. Clostridia_2	0.0(0.0-1.1)	0.0(0.0-0.7)	0.1(0.0-0.7)	0.0(0.0-0.0)
unclass. Clostridiales	0.1(0.0-0.3)	0.2(0.0-0.3)	0.2(0.1-0.3)	0.2(0.0-0.3)

Table 6. Continued.

	median(min-max) *in percent			
Taxa	**Healthy**	**AD**	**NHD**	**AHD**
Genus				
unclass. Clostridiaceae_1	0.0(0.0-2.7)	0.0(0.0-1.8)	0.2(0.0-1.8)	0.0(0.0-0.2)
unclass. Clostridiaceae_2	0.0(0.0-0.0)	0.0(0.0-1.2)	0.0(0.0-1.2)	0.0(0.0-0.0)
Clostridium	13.2(4.7-16.0)	31.2(0.3-53.8)	31.2(0.3-43.8)	31.8(17.2-53.8)
Sarcina	0.0(0.0-0.0)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.0(0.0-0.0)
unclass. Lachnospiraceae_1	4.7(3.2-6.7)	1.6(0.2-3.6)	2.9(0.9-3.6)	1.1(0.2-2.9)
unclass. Lachnospiraceae_2	0.4(0.0-0.6)	0.1(0.0-0.3)	0.1(0.1-0.2)	0.0(0.0-0.3)
Blautia	14.0(8.9-25.2)	3.6(0.2-19.8)	5.1(0.9-19.8)	2.7(0.2-17.0)
Coprococcus	0.0(0.0-1.7)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Dorea	1.5(0.3-12.6)	2.0(0.2-9.8)	2.4(0.2-9.8)	1.6(0.2-6.3)
Roseburia	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
[Ruminococcus]	2.7(0.7-10.6)	2.8(0.1-15.6)	2.8(0.3-15.6)	2.6(0.1-7.0)
Peptococcus	0.2(0.0-1.3)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.0(0.0-0.1)
unclass. Peptostreptococcaceae_1	0.0(0.0-0.0)	0.0(0.0-0.3)	0.0(0.0-0.0)	0.0(0.0-0.3)
unclass. Peptostreptococcaceae_2	0.3(0.1-5.7)	0.1(0.0-4.5)	0.1(0.0-3.5)	0.1(0.0-4.5)
unclass. Ruminococcaceae_1	0.1(0.0-0.3)	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.1)
unclass. Ruminococcaceae_2	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.1)
Faecalibacterium	1.5(0.1-5.4)	0.1(0.0-1.1)	0.0(0.0-1.1)	0.1(0.0-0.9)
Oscillospira	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Ruminococcus	0.1(0.0-0.2)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.1(0.0-0.3)
unclass. Veilonellaceae	0.1(0.0-1.1)	0.0(0.0-0.4)	0.0(0.0-0.2)	0.0(0.0-0.4)
Dialister	0.0(0.0-0.0)	0.0(0.0-0.4)	0.0(0.0-0.0)	0.0(0.0-0.4)
Megamonas	1.9(0.1-20.5)	0.6(0.0-1.9)	0.1(0.0-1.9)	0.9(0.2-1.8)
Megasphaera	0.0(0.0-0.0)	0.0(0.0-1.4)	0.0(0.0-0.0)	0.0(0.0-1.4)
Phascolarctobacterium	0.0(0.0-1.3)	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.2)

Table 6. Continued.

	median(min-max) *in percent			
Taxa	**Healthy**	**AD**	**NHD**	**AHD**
Genus				
Collinsella	0.3(0.1-1.7)	0.0(0.0-0.4)	0.0(0.0-0.4)	0.1(0.0-0.3)
Slackia	0.0(0.0-0.1)	0.0(0.0-0.2)	0.0(0.0-0.2)	0.0(0.0-0.2)
unclass. Erysipelotrichales_1	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. Erysipelotrichales_2	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. Erysipelotrichaceae	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Allobaculum	0.6(0.0-1.9)	0.1(0.0-5.7)	0.1(0.0-5.7)	0.1(0.0-0.6)
Bulleidia	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
[Eubacterium]	1.0(0.4-3.8)	0.1(0.0-1.0)	0.1(0.0-0.4)	0.2(0.0-1.0)
unclass. [Coprobacillaceae]_1	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.0)
unclass. [Coprobacillaceae]_2	0.7(0.0-2.6)	0.0(0.0-5.4)	0.0(0.0-5.4)	0.0(0.0-0.3)
Catenibacterium	0.3(0.0-13.2)	0.1(0.0-1.6)	0.0(0.0-0.9)	0.1(0.0-1.6)
Coprobacillus	0.0(0.0-0.6)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.0)
unclass. Fusobacteriaceae_1	0.0(0.0-0.3)	0.0(0.0-0.2)	0.2(0.0-0.2)	0.0(0.0-0.1)
unclass. Fusobacteriaceae_2	3.2(0.1-11.8)	12.0(0.0-44.0)	21.9(0.0-44.0)	11.9(0.1-20.6)
J2-30	0.8(0.0-2.8)	3.5(0.0-8.2)	4.3(0.0-7.1)	2.9(0.0-8.2)
Sutterella	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)
Campylobacter	0.0(0.0-0.0)	0.0(0.0-0.3)	0.0(0.0-0.3)	0.0(0.0-0.0)
Helicobacter	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.1)
unclass. Succinivibrionaceae	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. Enterobacteriaceae	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Erwinia	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.1)	0.0(0.0-0.0)
Escherichia	0.0(0.0-0.1)	0.0(0.0-0.5)	0.0(0.0-0.5)	0.0(0.0-0.0)
Providencia	0.0(0.0-0.0)	0.0(0.0-0.6)	0.0(0.0-0.6)	0.0(0.0-0.0)
unclass. Pasteurellaceae	0.0(0.0-0.0)	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.1)

Table 6. Continued.

	median(min-max) *in percent			
Taxa	**Healthy**	**AD**	**NHD**	**AHD**
Genus				
Azomonas	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
unclass. Mollicutes	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)
Anaeroplasma	0.0(0.0-0.0)	0.0(0.0-0.2)	0.0(0.0-0.0)	0.0(0.0-0.2)
unclass. RF39	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0(0.0-0.0)

NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea; AD = both groups combined (NHD and AHD).
The abbreviation “unclass.” denotes an unclassified taxonomy within respective taxonomic group. Brackets “[]” denote a proposed taxonomy that has not yet been verified.

Quantitative PCR. Quantitative PCR data are illustrated in Figure 6 and summarized in Table 7. *Blautia* spp. were significantly decreased in dogs with AD (log DNA median [range]: 9.8 [7.9-10.5]) compared to healthy dogs (log DNA median [range]: 10.5 [8.7-10.6]; p=0.0472). The abundance of *Clostridium perfringens* was significantly increased in dogs with AD (log DNA median [range]: 7 [5.8-7.5]) compared to healthy dogs (log DNA median [range]: 4.6 [3-6.1]; p=0.0088). The abundance of *Clostridium perfringens* was also significantly increased in dogs with AHD (log DNA median [range]: 7.1 [6.9-7.4]) compared to healthy dogs (log DNA median [range]: 4.6 [3-6.1]; Dunn's post-test, $p<0.05$). However, no statistical significance could be identified between animal groups for the abundance of the remaining bacterial groups or the gene for the coenzyme A activated form of butyric acid.

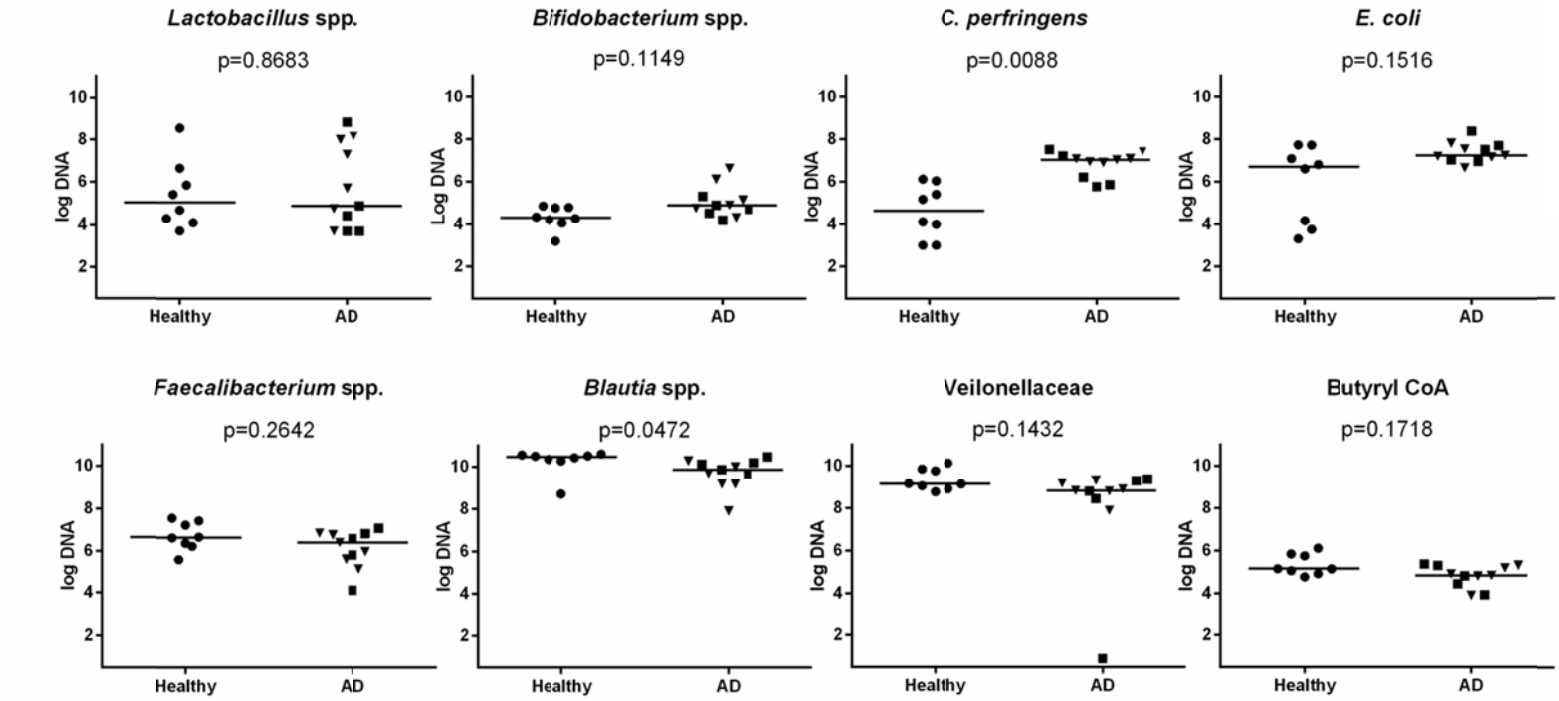

Figure 6. **Quantitative PCR results (log DNA).** Circles ● = healthy dogs; Squares ■ = dogs with acute non-hemorrhagic diarrhea; Triangles ▼ = dogs with acute hemorrhagic diarrhea. AD = both groups combined (NHD and AHD). Bars represent the median value for each group.

Table 7. Summary statistics of qPCR results.

	median (range) *log DNA					
Bacterial Groups	**Healthy**	**AD**	**NHD**	**AHD**	**M-W p-value**	**K-W p-value**
Bifidobacterium spp.	4.3(3.2-4.9)	4.9(4.2-6.6)	4.7(4.2-5.3)	5.0(4.3-6.6)	0.1149	0.1701
Lactobacillus spp.	5.0(3.7-8.5)	4.9(3.7-8.8)	4.4(3.7-8.8)	6.5(3.7-8.1)	0.8683	0.5371
E. coli	6.7(3.3-7.7)	7.3(6.7-8.4)	7.5(7.0-8.4)	7.2(6.7-7.8)	0.1516	0.3754
Clostridium perfringens	4.6[a] (3.0-6.1)	7.0(5.8-7.5)	6.2[a,b](5.8-7.5)	7.1[b](6.9-7.4)	**0.0088**	**0.0272**
Faecalibacterium spp.	6.7(5.6-7.6)	6.4(4.2-7.1)	6.6(4.2-7.1)	6.2(5.2-6.9)	0.2642	0.5069
Blautia spp.	10.5(8.7-10.6)	9.8(7.9-10.5)	10.1(9.6-10.5)	9.4(7.9-10.3)	**0.0472**	0.0836
Veilonellaceae	9.1(8.8-10.1)	8.8(0.9-9.4)	8.8(0.9-9.4)	8.9(7.9-9.3)	0.1432	0.4072
Butyryl CoA	5.1(4.8-6.1)	4.8(3.9-5.4)	4.8(3.9-5.4)	4.9(3.9-5.3)	0.1718	0.3409

NHD = dogs with acute non-hemorrhagic Diarrhea; AHD = dogs with acute hemorrhagic diarrhea; AD = both groups combined (NHD and AHD).
M-W p-value = P-value from Mann-Whitney U test comparing healthy dogs vs. AD adjusted based on the Benjamini and Hochberg False Discovery Rate.
K-W p-value = P-value from Kruskal-Wallis test comparing healthy dogs vs. NHD vs. AHD adjusted based on the Benjamini and Hochberg False Discovery Rate.
Medians not sharing a common superscript are significantly different ($p<0.05$ based on Dunn's post-test).

Microbial communities. PCoA plots (Figure 7) based on unweighted Unifrac distances were constructed to compare groups of dogs, and showed significant differences between healthy dogs and dogs with AD (ANOSIM; healthy dogs vs. dogs with AD, $p = 0.0040$). Furthermore, dogs with NHD and dogs with AHD differed significantly from healthy dogs (ANOSIM; dogs with NHD and dogs with AHD vs. healthy dogs, $p = 0.0020$ for both).

Linear Discriminant Analysis Effect Size (LEfSe). LEfse is an algorithm for high-dimensional biomarker discovery and is an explanation that identifies genomic features (genes, pathways, or taxa) characterizing the differences between two or more biological conditions (e.g., healthy dogs and dogs with acute diarrhea). LEfSe emphasizes both statistical significance and biological relevance, allowing the identification of differentially abundant bacterial groups that are also consistent with biologically meaningful categories. *Clostridium* spp. were discovered as high-dimensional biomarkers for explaining effects in dogs with AD. In contrast, *Prevotella* spp., *Blautia* spp., *Faecalibacterium* spp., *Eubacterium* spp., an unclassified genus within the families Ruminococcaceae, Lachnospiraceae, Clostridia, Ruminococcaceae, and Coprobacillaceae were discovered as high-dimensional biomarkers for explaining effects in healthy dogs (Figure 8).

Heat-map illustrating relative sequence abundances. The results in Figure 9 illustrate a decrease in predominant bacterial groups (e.g., *Faecalibacterium* and an unclassified genus in Ruminococcaceae) and increase in sequences belonging to *Clostridium* in dogs with NHD and dogs with AHD, respectively.

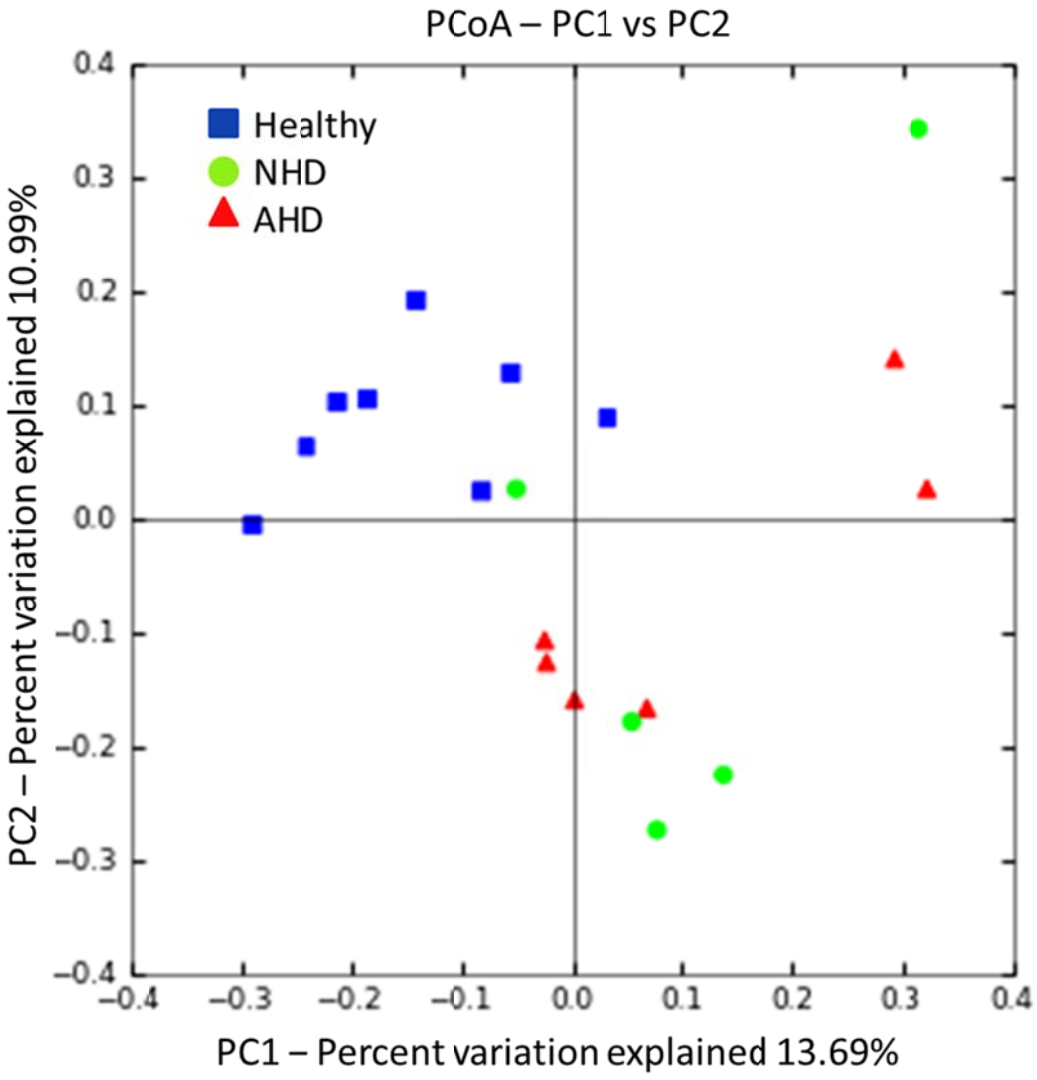

Figure 7. Principal Coordinate Analysis (PCoA) of unweighted UniFrac distances of 16S rRNA genes. Blue squares = healthy dogs, green circles = dogs with acute non-hemorrhagic diarrhea (NHD), and red triangles = dogs with acute hemorrhagic diarrhea (AHD); (ANOSIM for healthy dogs vs. dogs with AD, p=0.0040; and ANOSIM for dogs with NHD and dogs with AHD vs. healthy dog, p=0.0020 for both).

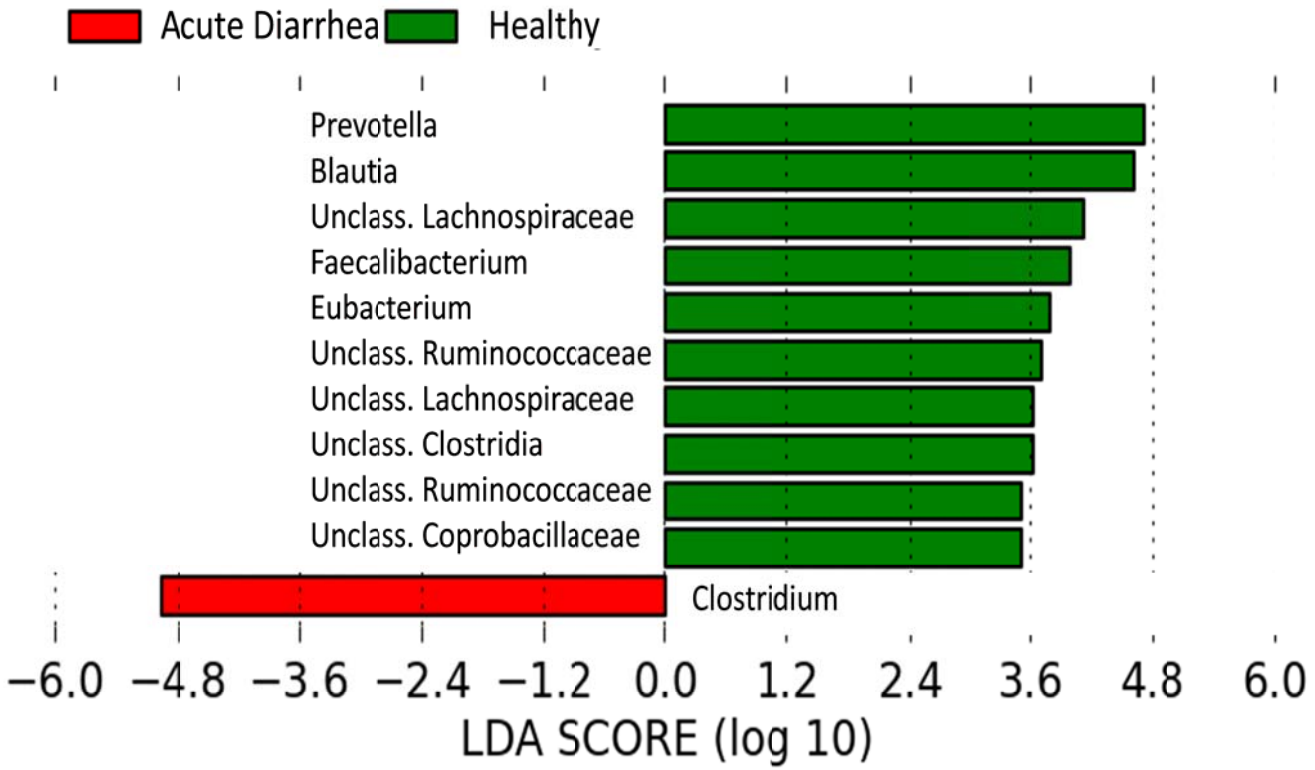

Figure 8. **Linear Discriminant Analysis Effect Size (LEfSe).** Histogram of the linear discriminant analysis (LDA) scores computed for features differentially expressed between healthy dogs and dogs with acute diarrhea. LEfSe scores can be interpreted as the degree of consistent difference in relative abundance between features in the two groups of analyzed microbial communities. The histogram thus identifies which taxa among all those detected are statistically and biologically differential explaining the greatest differences between communities. Red bars represent bacterial groups significantly associated with dogs with AD, while green bars represent bacterial groups significantly associated with healthy dogs. The abbreviation "unclass." denotes an unclassified taxonomy within respective taxonomic families

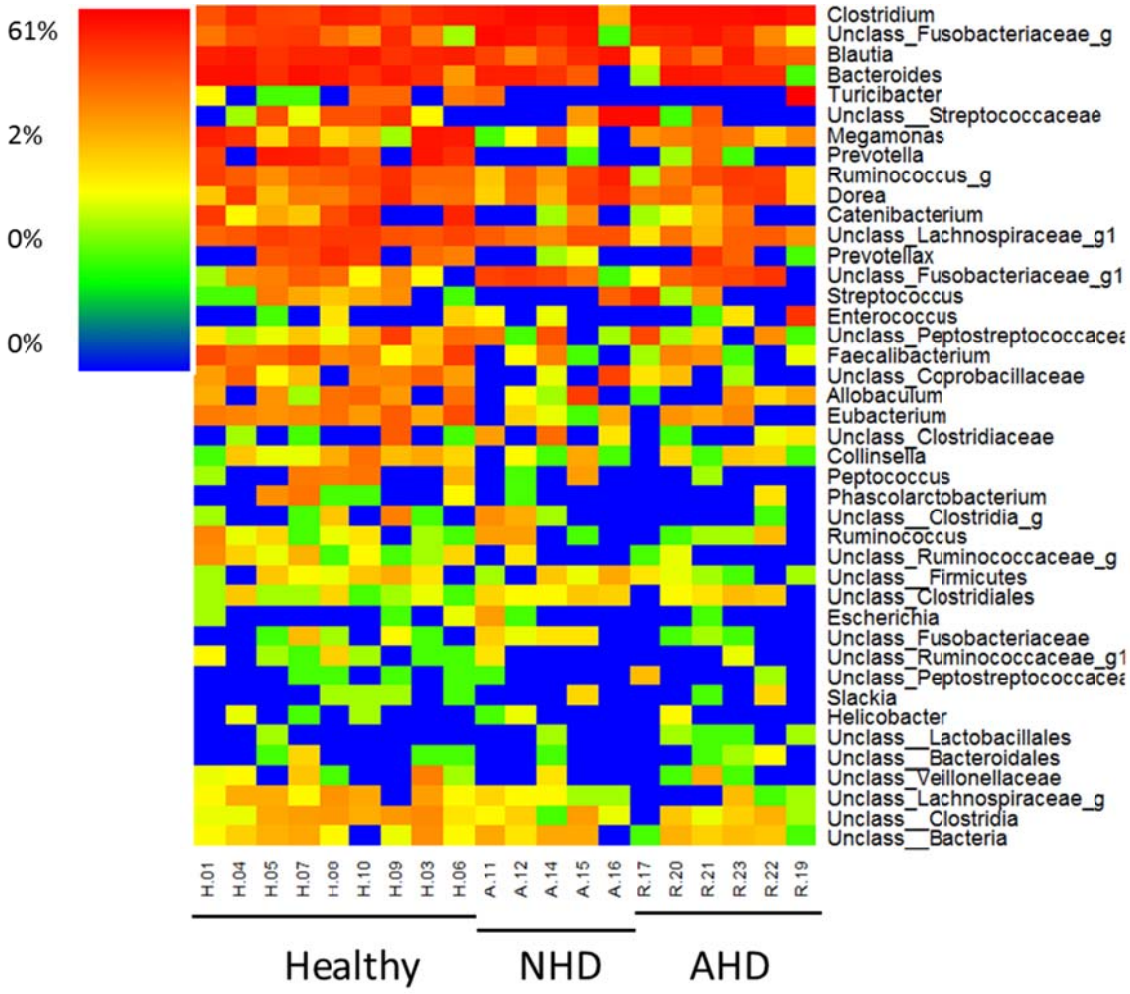

Figure 9. **Heatmap illustrating the relative abundance of predominant bacterial genera in fecal samples.** Healthy = healthy dogs; NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea; based on 454-pyrosequencing. The heatmap represents the relative percentages of each genus within each sample.

Functional gene categories. The functional gene content of the fecal microbiome was predicted from the 16S rRNA data using the online software Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), and compared between healthy dogs and dogs with AD (Table 8, 9, and 10). Figure 10 shows a comparison of taxonomic phyla (Figure 10A) and functional gene category (Figure 10B) variations in the canine fecal microbiome. The area chart in Figure 10B revealed a generally consistent number of KEGG orthologs belonging to functional gene categories across all dogs. There were no significant differences in the number of KEGG orthologs belonging to functional gene categories at all levels (e.g., 1, 2, and 3) among all groups of dogs after correcting for multiple comparisons using the Benjamini and Hochberg False Discovery Rate.

SCFA. There were no significant differences in total SCFA or BCFA concentrations (μmol/g of dry feces) observed between groups of dogs (Figure 11). Summary statistics for these results are shown in Table 11.

In a separate analysis, butyric acid, acetic acid, and propionic acid were each expressed as a percentage of the total amount of fecal SCFA concentrations (Figure 12). The proportion of propionic acid was significantly decreased in dogs with AD (median [range]:12% [0-25%]) compared to healthy dogs (median [range]: 30% [20-44%]; p=0.0033). In contrast, the proportion of butyric acid was significantly increased in dogs with AD (median [range]: 12% [8-26%]) compared to healthy dogs (median [range]: 6% [4-8%]; p=0.0048). Summary statistics for these results are shown in Table 12.

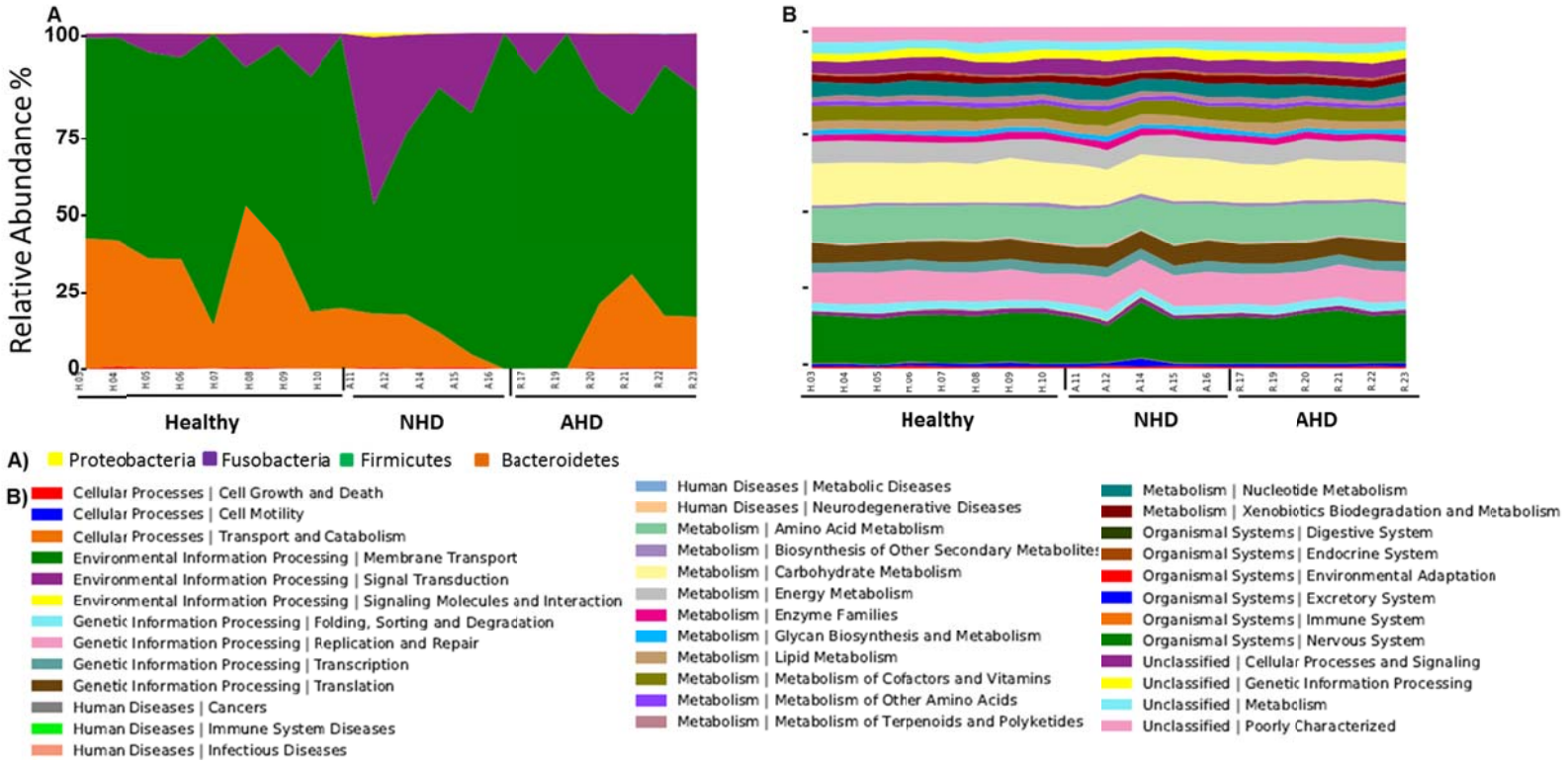

Figure 10. Comparison of taxonomic and functional variations in the canine fecal microbiome. A) Area chart of sequence percentages belonging to phyla among all dogs. B) Area chart of KEGG ortholog percentages belonging to functional gene categories at level 2 among all dogs. Healthy = healthy dogs; NHD = dogs with acute non-hemorrhagic diarrhea; AHD = dogs with acute hemorrhagic diarrhea.

Table 8. Relative percentages of KEGG orthologs belonging to functional gene categories at Level 1.

	Median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Cellular Processes	1.7(1.4-1.8)	1.5(1.3-3.6)	0.4556	0.9112
Environmental Information Processing	15.3(14.7-16.4)	15.1(12.5-17.1)	0.8364	0.9559
Genetic Information Processing	19.7(19.1-20.4)	19.8(19.0-22.1)	0.4824	0.7718
Human Diseases	0.6(0.6-0.7)	0.7(0.4-0.8)	0.5589	0.7452
Metabolism	49.3(47.5-49.5)	48.7(46.1-49.5)	0.3627	0.9672
None	0.1(0.0-0.2)	0.1(0.0-0.2)	0.1436	1.0000
Organismal Systems	0.6(0.5-0.9)	0.6(0.5-0.8)	0.9339	0.9339
Unclassified	13.2(12.8-13.6)	13.5(12.7-14.0)	0.2306	0.9224

M-W p-value = P-value from Mann-Whitney U test comparing healthy dogs and dogs with AD.
M-W FDR p-value = P-value from Mann-Whitney U test that is adjusted based on the Benjamini and Hochberg False Discovery Rate.

Table 9. Relative percentages of KEGG orthologs belonging to functional gene categories at level 2.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Cellular Processes | Cell Growth and Death	0.5(0.4-0.9)	0.5(0.5-0.7)	0.8354	0.9659
Cellular Processes | Cell Motility	0.8(0.6-1.2)	0.9(0.7-2.4)	0.3618	0.8924
Cellular Processes | Transport and Catabolism	0.2(0.0-0.2)	0.1(0.1-0.2)	0.5238	0.9229
Environmental Information Processing | Membrane Transport	14.1(13.6-15.0)	13.7(10.9-16.2)	0.3421	0.9041
Environmental Information Processing | Signal Transduction	1.3(0.9-1.6)	1.2(1.0-1.6)	0.3406	0.9694
Environmental Information Processing | Signaling Molecules and Interaction	0.1(0.1-0.2)	0.1(0.0-0.2)	0.2956	0.9114
Genetic Information Processing | Folding, Sorting and Degradation	2.3(2.1-2.6)	2.4(2.2-2.7)	0.1996	1.0000
Genetic Information Processing | Replication and Repair	9.0(7.9-9.5)	9.0(8.5-10.0)	0.2475	0.9158
Genetic Information Processing | Transcription	3.1(2.8-3.3)	3.0(2.7-3.3)	0.9340	0.9874
Genetic Information Processing | Translation	5.9(5.0-6.3)	5.7(5.0-6.2)	0.5905	0.8403
Human Diseases | Cancers	0.0(0.0-0.2)	0.1(0.0-0.1)	0.5570	0.8960
Human Diseases | Immune System Diseases	0.0(0.0-0.1)	0.1(0.0-0.1)	0.5833	0.8633
Human Diseases | Infectious Diseases	0.3(0.2-0.4)	0.3(0.2-0.5)	0.7390	0.9429
Human Diseases | Metabolic Diseases	0.1(0.0-0.2)	0.1(0.0-0.2)	0.7383	0.9756
Human Diseases | Neurodegenerative Diseases	0.0(0.0-0.1)	0.0(0.0-0.1)	0.2246	1.0000
Metabolism | Amino Acid Metabolism	10.0(9.2-10.8)	10.6(9.2-11.8)	0.1485	1.0000
Metabolism | Biosynthesis of Other Secondary Metabolites	1.0(0.8-1.4)	1.0(0.8-1.3)	0.2456	1.0000
Metabolism | Carbohydrate Metabolism	11.6(11.1-13.0)	11.3(10.1-12.7)	0.1863	1.0000
Metabolism | Energy Metabolism	6.3(5.4-6.8)	5.8(5.4-6.6)	0.2151	1.0000
Metabolism | Enzyme Families	2.1(1.8-2.3)	2.0(1.7-2.4)	0.4320	0.8880
Metabolism | Glycan Biosynthesis and Metabolism	1.4(1.1-1.7)	1.3(1.1-1.9)	0.9670	0.9670
Metabolism | Lipid Metabolism	2.6(2.3-2.9)	2.6(2.1-3.2)	0.7720	0.9521
Metabolism | Metabolism of Cofactors and Vitamins	4.5(3.6-4.7)	4.3(4.0-4.8)	0.9341	0.9600
Metabolism | Metabolism of Other Amino Acids	1.4(1.1-1.6)	1.3(1.0-1.4)	0.0895	1.0000

Table 9. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Metabolism | Metabolism of Terpenoids and Polyketides	1.6(1.3-1.8)	1.6(1.1-1.8)	0.5076	0.9391
Metabolism | Nucleotide Metabolism	3.9(3.4-4.6)	3.9(3.5-4.7)	0.6494	0.8899
Metabolism | Xenobiotics Biodegradation and Metabolism	2.1(1.6-2.3)	2.3(1.8-2.6)	**0.0472**	1.0000
Organismal Systems | Digestive System	0.0(0.0-0.0)	0.0(0.0-0.0)	0.5771	0.8897
Organismal Systems | Endocrine System	0.2(0.2-0.3)	0.2(0.1-0.4)	0.7986	0.9532
Organismal Systems | Environmental Adaptation	0.2(0.1-0.3)	0.2(0.1-0.2)	0.8671	0.9722
Organismal Systems | Excretory System	0.0(0.0-0.0)	0.0(0.0-0.1)	0.5351	0.8999
Organismal Systems | Immune System	0.1(0.0-0.2)	0.1(0.1-0.2)	0.0768	1.0000
Organismal Systems | Nervous System	0.1(0.1-0.1)	0.1(0.0-0.2)	0.3735	0.8637
Unclassified | Cellular Processes and Signaling	3.9(3.5-4.4)	4.0(3.6-4.6)	0.3857	0.8395
Unclassified | Genetic Information Processing	2.4(2.1-3.0)	2.3(2.2-3.1)	0.9013	0.9808
Unclassified | Metabolism	2.9(2.3-3.2)	2.7(2.2-3.0)	0.2650	0.8914
Unclassified | Poorly Characterized	4.2(3.9-4.8)	4.3(4.0-4.9)	0.4820	0.9386

M-W p-value = P-value from Mann-Whitney U test comparing healthy dogs and dogs with AD.
M-W FDR p-value=P-value from Mann-Whitney U test that is adjusted based on the Benjamini and Hochberg False Discovery Rate.

Table 10. Relative percentages of KEGG orthologs belonging to functional gene categories at Level 3.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Cellular Processes | Cell Growth and Death | Cell cycle - Caulobacter	0.6(0.3-0.9)	0.5(0.4-0.8)	0.4816	0.9767
Cellular Processes | Cell Motility | Bacterial chemotaxis	0.3(0.2-0.4)	0.2(0.1-0.6)	0.9338	1.0000
Cellular Processes | Cell Motility | Bacterial motility proteins	0.2(0.0-0.2)	0.2(0.0-1.0)	0.2599	1.0000
Cellular Processes | Cell Motility | Cytoskeleton proteins	0.4(0.2-0.5)	0.4(0.3-0.6)	0.5337	0.9815
Cellular Processes | Cell Motility | Flagellar assembly	0.1(0.1-0.2)	0.1(0.0-0.2)	0.7574	1.0000
Cellular Processes | Transport and Catabolism | Lysosome	4.2(3.4-4.4)	4.3(2.6-4.9)	1.0000	1.0000
Cellular Processes | Transport and Catabolism | Peroxisome	0.5(0.4-0.6)	0.4(0.2-0.7)	0.1231	1.0000
Environmental Information Processing | Membrane Transport | ABC transporters	0.4(0.2-2.3)	0.4(0.2-1.3)	0.3421	0.9768
Environmental Information Processing | Membrane Transport | Bacterial secretion system	0.8(0.7-1.0)	0.9(0.8-1.1)	0.3601	0.9647
Environmental Information Processing | Membrane Transport | Phosphotransferase system (PTS)	7.9(7.4-8.7)	8.1(6.0-8.9)	0.8685	1.0000
Environmental Information Processing | Membrane Transport | Secretion system	0.0(0.0-0.1)	0.0(0.0-0.1)	0.3200	1.0000
Environmental Information Processing | Membrane Transport | Transporters	0.1(0.0-0.2)	0.1(0.0-0.1)	0.9014	1.0000
Environmental Information Processing | Signal Transduction | MAPK signaling pathway - yeast	1.1(0.7-1.4)	1.1(0.8-1.5)	0.8645	1.0000
Environmental Information Processing | Signal Transduction | Phosphatidylinositol signaling system	0.1(0.1-0.2)	0.1(0.1-0.2)	1.0000	1.0000
Environmental Information Processing | Signal Transduction | Two-component system	0.9(0.6-1.1)	0.9(0.7-1.2)	0.8363	1.0000
Environmental Information Processing | Signaling Molecules and Interaction | Bacterial toxins	0.0(0.0-0.1)	0.0(0.0-0.1)	0.3284	1.0000
Environmental Information Processing | Signaling Molecules and Interaction | Cellular antigens	0.5(0.4-0.7)	0.5(0.3-0.7)	0.4742	0.9894
Environmental Information Processing | Signaling Molecules and Interaction | Ion channels	0.1(0.0-0.1)	0.0(0.0-0.1)	0.4835	0.9715
Genetic Information Processing | Folding, Sorting and Degradation | Chaperones and folding catalysts	0.4(0.2-0.5)	0.5(0.2-0.7)	0.4806	0.9839
Genetic Information Processing | Folding, Sorting and Degradation | Proteasome	0.2(0.2-0.4)	0.3(0.3-0.5)	0.5528	0.9914
Genetic Information Processing | Folding, Sorting and Degradation | Protein export	0.5(0.3-0.6)	0.5(0.4-0.6)	0.7394	1.0000
Genetic Information Processing | Folding, Sorting and Degradation | Protein processing in endoplasmic reticulum	1.5(1.4-1.7)	1.5(1.3-1.9)	0.7353	1.0000
Genetic Information Processing | Folding, Sorting and Degradation | RNA degradation	3.0(2.7-3.3)	2.8(2.6-3.4)	0.0675	1.0000
Genetic Information Processing | Folding, Sorting and Degradation | Sulfur relay system	0.7(0.5-0.8)	0.7(0.5-0.8)	**0.0079**	0.8572
Genetic Information Processing | Replication and Repair | Base excision repair	1.2(0.9-1.5)	1.3(1.0-1.7)	0.7383	1.0000

Table 10. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Genetic Information Processing | Replication and Repair | Chromosome	1.0(0.7-1.2)	1.0(0.7-1.2)	0.7095	1.0000
Genetic Information Processing | Replication and Repair | DNA repair and recombination proteins	0.8(0.7-1.0)	0.8(0.7-1.0)	0.1998	1.0000
Genetic Information Processing | Replication and Repair | DNA replication	0.4(0.2-0.5)	0.4(0.2-0.5)	1.0000	1.0000
Genetic Information Processing | Replication and Repair | DNA replication proteins	0.2(0.1-0.2)	0.2(0.0-0.2)	0.3203	0.9929
Genetic Information Processing | Replication and Repair | Homologous recombination	2.1(1.4-2.3)	2.1(1.7-2.3)	0.7720	1.0000
Genetic Information Processing | Replication and Repair | Mismatch repair	1.0(0.8-1.2)	1.0(0.9-1.1)	0.6480	1.0000
Genetic Information Processing | Replication and Repair | Non-homologous end-joining	1.2(1.0-1.5)	1.1(0.8-1.6)	0.2864	1.0000
Genetic Information Processing | Replication and Repair | Nucleotide excision repair	0.2(0.1-0.2)	0.2(0.0-0.2)	0.1322	1.0000
Genetic Information Processing | Transcription | Basal transcription factors	2.2(2.0-2.6)	2.3(1.7-2.6)	0.4555	1.0000
Genetic Information Processing | Transcription | RNA polymerase	1.4(1.1-1.7)	1.3(1.0-1.6)	0.2321	0.9686
Genetic Information Processing | Transcription | Transcription factors	0.0(0.0-0.1)	0.0(0.0-0.1)	0.8680	1.0000
Genetic Information Processing | Transcription | Transcription machinery	0.6(0.5-0.7)	0.5(0.4-0.9)	0.8674	1.0000
Genetic Information Processing | Translation | Aminoacyl-tRNA biosynthesis	0.0(0.0-0.1)	0.0(0.0-0.1)	0.8687	1.0000
Genetic Information Processing | Translation | Ribosome	0.0(0.0-0.1)	0.0(0.0-0.1)	0.8362	1.0000
Genetic Information Processing | Translation | Ribosome Biogenesis	0.0(0.0-0.1)	0.0(0.0-0.1)	0.5348	0.9752
Genetic Information Processing | Translation | Ribosome biogenesis in eukaryotes	0.1(0.0-0.2)	0.1(0.0-0.2)	0.9659	1.0000
Genetic Information Processing | Translation | RNA transport	0.1(0.1-0.2)	0.1(0.1-0.2)	0.1695	1.0000
Genetic Information Processing | Translation | Translation factors	0.1(0.0-0.1)	0.1(0.0-0.2)	0.1587	1.0000
Human Diseases | Cancers | Pathways in cancer	0.0(0.0-0.1)	0.0(0.0-0.1)	0.4552	1.0000
Human Diseases | Cancers | Prostate cancer	0.0(0.0-0.1)	0.0(0.0-0.1)	0.6601	1.0000
Human Diseases | Immune System Diseases | Primary immunodeficiency	0.0(0.0-0.1)	0.1(0.0-0.1)	0.5487	0.9922
Human Diseases | Infectious Diseases | Epithelial cell signaling in Helicobacter pylori infection	1.2(0.9-1.3)	1.1(0.9-1.5)	0.8999	1.0000
Human Diseases | Infectious Diseases | Pertussis	1.4(1.2-1.6)	1.5(1.3-1.6)	0.2864	1.0000
Human Diseases | Infectious Diseases | Staphylococcus aureus infection	1.3(1.1-1.5)	1.3(1.2-1.5)	0.7940	1.0000
Human Diseases | Infectious Diseases | Tuberculosis	0.9(0.6-1.2)	1.0(0.8-1.3)	0.3573	0.9814

Table 10. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Human Diseases | Infectious Diseases | Vibrio cholerae pathogenic cycle	0.9(0.8-1.0)	0.9(0.7-1.1)	0.5834	1.0000
Human Diseases | Metabolic Diseases | Type I diabetes mellitus	0.8(0.6-1.0)	0.7(0.5-1.0)	0.8653	1.0000
Human Diseases | Metabolic Diseases | Type II diabetes mellitus	0.8(0.7-1.0)	0.9(0.7-1.1)	0.4605	0.9702
Human Diseases | Neurodegenerative Diseases | Alzheimer's disease	0.1(0.0-0.2)	0.1(0.1-0.2)	0.4743	0.9802
Human Diseases | Neurodegenerative Diseases | Huntington's disease	0.2(0.1-0.3)	0.2(0.1-0.3)	0.8617	1.0000
Human Diseases | Neurodegenerative Diseases | Parkinson's disease	0.9(0.8-1.2)	0.9(0.7-1.2)	0.4555	1.0000
Metabolism | Amino Acid Metabolism | Alanine, aspartate and glutamate metabolism	0.1(0.1-0.2)	0.1(0.0-0.2)	0.3192	1.0000
Metabolism | Amino Acid Metabolism | Amino acid related enzymes	0.5(0.3-0.6)	0.6(0.3-0.7)	0.8683	1.0000
Metabolism | Amino Acid Metabolism | Arginine and proline metabolism	0.7(0.5-1.0)	0.7(0.6-0.9)	0.4301	1.0000
Metabolism | Amino Acid Metabolism | Cysteine and methionine metabolism	0.2(0.1-0.2)	0.2(0.1-0.2)	0.2988	0.9975
Metabolism | Amino Acid Metabolism | Glycine, serine and threonine metabolism	0.1(0.1-0.1)	0.1(0.0-0.2)	0.9011	1.0000
Metabolism | Amino Acid Metabolism | Histidine metabolism	0.1(0.0-0.1)	0.1(0.0-0.1)	0.2147	1.0000
Metabolism | Amino Acid Metabolism | Lysine biosynthesis	0.2(0.2-0.3)	0.2(0.1-0.3)	0.1246	1.0000
Metabolism | Amino Acid Metabolism | Lysine degradation	0.1(0.1-0.2)	0.1(0.0-0.2)	0.7068	1.0000
Metabolism | Amino Acid Metabolism | Phenylalanine metabolism	0.3(0.2-0.5)	0.2(0.1-0.4)	0.7711	1.0000
Metabolism | Amino Acid Metabolism | Phenylalanine, tyrosine and tryptophan biosynthesis	0.2(0.1-0.3)	0.2(0.0-0.3)	0.7096	1.0000
Metabolism | Amino Acid Metabolism | Tryptophan metabolism	0.0(0.0-0.1)	0.0(0.0-0.1)	0.5544	0.9861
Metabolism | Amino Acid Metabolism | Tyrosine metabolism	1.4(0.9-2.2)	1.3(1.0-1.7)	0.9007	1.0000
Metabolism | Amino Acid Metabolism | Valine, leucine and isoleucine biosynthesis	0.1(0.0-0.2)	0.1(0.0-0.1)	0.6487	1.0000
Metabolism | Amino Acid Metabolism | Valine, leucine and isoleucine degradation	0.7(0.5-0.9)	0.7(0.5-0.8)	0.8332	1.0000
Metabolism | Biosynthesis of Other Secondary Metabolites | beta-Lactam resistance	0.4(0.2-0.6)	0.4(0.3-0.7)	0.1969	1.0000
Metabolism | Biosynthesis of Other Secondary Metabolites | Butirosin and neomycin biosynthesis	0.5(0.4-0.8)	0.6(0.1-0.8)	0.8649	1.0000
Metabolism | Biosynthesis of Other Secondary Metabolites | Flavone and flavonol biosynthesis	1.3(1.0-1.8)	1.2(1.0-1.5)	0.4555	1.0000
Metabolism | Biosynthesis of Other Secondary Metabolites | Isoquinoline alkaloid biosynthesis	0.7(0.6-0.9)	0.7(0.5-0.9)	0.4155	1.0000
Metabolism | Biosynthesis of Other Secondary Metabolites | Novobiocin biosynthesis	1.3(1.1-1.5)	1.3(1.1-1.6)	0.2227	1.0000

Table 10. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Metabolism | Biosynthesis of Other Secondary Metabolites | Phenylpropanoid biosynthesis	0.5(0.4-0.7)	0.5(0.4-0.8)	0.1104	0.9982
Metabolism | Biosynthesis of Other Secondary Metabolites | Stilbenoid, diarylheptanoid and gingerol biosynthesis	0.1(0.0-0.2)	0.1(0.0-0.1)	0.4555	0.9984
Metabolism | Biosynthesis of Other Secondary Metabolites | Streptomycin biosynthesis	0.6(0.5-0.7)	0.6(0.2-0.7)	0.1712	1.0000
Metabolism | Biosynthesis of Other Secondary Metabolites | Tropane, piperidine and pyridine alkaloid biosynthesis	1.0(0.9-1.4)	1.2(0.9-1.3)	0.3387	1.0000
Metabolism | Carbohydrate Metabolism | Amino sugar and nucleotide sugar metabolism	0.5(0.4-0.8)	0.6(0.4-0.8)	0.4322	1.0000
Metabolism | Carbohydrate Metabolism | Ascorbate and aldarate metabolism	1.1(0.9-1.3)	1.1(0.8-1.3)	0.1027	1.0000
Metabolism | Carbohydrate Metabolism | Butanoate metabolism	1.2(1.0-1.5)	1.2(0.9-1.3)	0.3597	0.9757
Metabolism | Carbohydrate Metabolism | C5-Branched dibasic acid metabolism	0.8(0.5-1.0)	0.7(0.6-0.9)	0.6194	1.0000
Metabolism | Carbohydrate Metabolism | Citrate cycle (TCA cycle)	0.9(0.8-1.2)	1.0(0.7-1.1)	0.4303	1.0000
Metabolism | Carbohydrate Metabolism | Fructose and mannose metabolism	1.5(1.3-1.9)	1.5(1.2-2.2)	0.1061	1.0000
Metabolism | Carbohydrate Metabolism | Galactose metabolism	0.6(0.5-0.9)	0.7(0.4-0.8)	0.2300	0.9982
Metabolism | Carbohydrate Metabolism | Glycolysis / Gluconeogenesis	1.2(0.9-1.5)	1.3(0.8-1.4)	0.2298	1.0000
Metabolism | Carbohydrate Metabolism | Glyoxylate and dicarboxylate metabolism	0.5(0.4-0.7)	0.4(0.3-0.6)	1.0000	1.0000
Metabolism | Carbohydrate Metabolism | Inositol phosphate metabolism	0.4(0.2-0.6)	0.4(0.2-0.6)	1.0000	1.0000
Metabolism | Carbohydrate Metabolism | Pentose and glucuronate interconversions	0.2(0.1-0.4)	0.2(0.1-0.3)	0.2108	1.0000
Metabolism | Carbohydrate Metabolism | Pentose phosphate pathway	1.9(1.6-2.1)	1.7(1.4-2.1)	0.5332	1.0000
Metabolism | Carbohydrate Metabolism | Propanoate metabolism	0.2(0.2-0.4)	0.3(0.1-0.5)	0.0961	1.0000
Metabolism | Carbohydrate Metabolism | Pyruvate metabolism	0.0(0.0-0.1)	0.1(0.0-0.1)	1.0000	1.0000
Metabolism | Carbohydrate Metabolism | Starch and sucrose metabolism	0.3(0.2-0.4)	0.2(0.1-0.5)	0.4075	1.0000
Metabolism | Energy Metabolism | Carbon fixation in photosynthetic organisms	0.1(0.0-0.1)	0.1(0.0-0.2)	0.0881	1.0000
Metabolism | Energy Metabolism | Carbon fixation pathways in prokaryotes	0.1(0.0-0.1)	0.1(0.0-0.2)	0.8360	1.0000
Metabolism | Energy Metabolism | Methane metabolism	1.0(0.8-1.1)	0.8(0.7-1.2)	0.8360	1.0000
Metabolism | Energy Metabolism | Nitrogen metabolism	0.1(0.1-0.2)	0.1(0.0-0.2)	0.9670	1.0000
Metabolism | Energy Metabolism | Oxidative phosphorylation	0.5(0.3-0.7)	0.6(0.3-0.8)	0.7714	1.0000
Metabolism | Energy Metabolism | Photosynthesis	0.2(0.2-0.4)	0.2(0.1-0.3)	0.1714	1.0000

Table 10. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Metabolism | Energy Metabolism | Photosynthesis proteins	0.4(0.4-0.7)	0.5(0.3-0.8)	0.9340	1.0000
Metabolism | Energy Metabolism | Sulfur metabolism	0.5(0.4-0.7)	0.5(0.4-0.6)	0.5323	1.0000
Metabolism | Enzyme Families | Peptidases	0.1(0.0-0.2)	0.1(0.0-0.2)	0.2308	0.9820
Metabolism | Enzyme Families | Protein kinases	0.5(0.3-0.7)	0.5(0.3-0.6)	0.3381	1.0000
Metabolism | Glycan Biosynthesis and Metabolism | Glycosaminoglycan degradation	0.0(0.0-0.1)	0.0(0.0-0.1)	0.6514	1.0000
Metabolism | Glycan Biosynthesis and Metabolism | Glycosphingolipid biosynthesis - ganglio series	0.0(0.0-0.1)	0.1(0.0-0.1)	0.1345	0.9729
Metabolism | Glycan Biosynthesis and Metabolism | Glycosphingolipid biosynthesis - globo series	0.1(0.0-0.1)	0.1(0.0-0.1)	**0.0068**	1.0000
Metabolism | Glycan Biosynthesis and Metabolism | Glycosyltransferases	0.1(0.1-0.3)	0.1(0.0-0.2)	0.2622	1.0000
Metabolism | Glycan Biosynthesis and Metabolism | Lipopolysaccharide biosynthesis	0.4(0.3-0.6)	0.4(0.2-0.5)	0.8171	1.0000
Metabolism | Glycan Biosynthesis and Metabolism | Lipopolysaccharide biosynthesis proteins	0.0(0.0-0.0)	0.0(0.0-0.0)	0.7034	1.0000
Metabolism | Glycan Biosynthesis and Metabolism | N-Glycan biosynthesis	0.4(0.4-0.6)	0.4(0.2-0.7)	0.1917	1.0000
Metabolism | Glycan Biosynthesis and Metabolism | Other glycan degradation	0.6(0.5-0.6)	0.6(0.4-0.7)	0.5819	1.0000
Metabolism | Glycan Biosynthesis and Metabolism | Peptidoglycan biosynthesis	0.5(0.4-0.7)	0.6(0.3-0.7)	0.0681	1.0000
Metabolism | Lipid Metabolism | Arachidonic acid metabolism	1.0(0.7-1.5)	1.2(1.0-1.4)	0.8163	1.0000
Metabolism | Lipid Metabolism | Biosynthesis of unsaturated fatty acids	0.1(0.0-0.1)	0.1(0.0-0.1)	0.7036	1.0000
Metabolism | Lipid Metabolism | Ether lipid metabolism	0.2(0.1-0.4)	0.3(0.2-0.5)	0.6695	1.0000
Metabolism | Lipid Metabolism | Fatty acid biosynthesis	0.5(0.3-0.7)	0.5(0.3-0.7)	0.7091	1.0000
Metabolism | Lipid Metabolism | Fatty acid metabolism	0.2(0.1-0.2)	0.2(0.1-0.3)	0.9665	1.0000
Metabolism | Lipid Metabolism | Glycerolipid metabolism	0.2(0.2-0.4)	0.2(0.1-0.4)	0.3402	0.9843
Metabolism | Lipid Metabolism | Glycerophospholipid metabolism	0.1(0.0-0.2)	0.1(0.1-0.2)	0.8020	1.0000
Metabolism | Lipid Metabolism | Linoleic acid metabolism	0.1(0.0-0.2)	0.1(0.1-0.3)	0.4012	1.0000
Metabolism | Lipid Metabolism | Lipid biosynthesis proteins	0.1(0.0-0.2)	0.1(0.1-0.4)	0.4564	0.9710
Metabolism | Lipid Metabolism | Primary bile acid biosynthesis	0.0(0.0-0.1)	0.0(0.0-0.1)	0.8994	1.0000
Metabolism | Lipid Metabolism | Secondary bile acid biosynthesis	0.2(0.1-0.3)	0.2(0.2-0.4)	0.1085	1.0000
Metabolism | Lipid Metabolism | Sphingolipid metabolism	0.1(0.0-0.2)	0.1(0.0-0.1)	0.7977	1.0000

Table 10. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Metabolism | Lipid Metabolism | Steroid hormone biosynthesis	0.2(0.2-0.3)	0.2(0.1-0.5)	0.4555	0.9884
Metabolism | Lipid Metabolism | Synthesis and degradation of ketone bodies	0.1(0.0-0.2)	0.1(0.1-0.2)	0.4045	1.0000
Metabolism | Metabolism of Cofactors and Vitamins | Biotin metabolism	0.0(0.0-0.1)	0.0(0.0-0.1)	0.2123	1.0000
Metabolism | Metabolism of Cofactors and Vitamins | Folate biosynthesis	0.2(0.0-0.3)	0.2(0.0-0.3)	0.5336	0.9897
Metabolism | Metabolism of Cofactors and Vitamins | Lipoic acid metabolism	0.1(0.0-0.3)	0.2(0.1-0.3)	1.0000	1.0000
Metabolism | Metabolism of Cofactors and Vitamins | Nicotinate and nicotinamide metabolism	0.2(0.1-0.4)	0.2(0.1-0.4)	1.0000	1.0000
Metabolism | Metabolism of Cofactors and Vitamins | One carbon pool by folate	0.5(0.4-0.7)	0.5(0.4-0.7)	0.5559	0.9807
Metabolism | Metabolism of Cofactors and Vitamins | Pantothenate and CoA biosynthesis	0.2(0.1-0.2)	0.2(0.1-0.3)	0.7401	1.0000
Metabolism | Metabolism of Cofactors and Vitamins | Porphyrin and chlorophyll metabolism	0.1(0.0-0.1)	0.0(0.0-0.1)	0.1477	1.0000
Metabolism | Metabolism of Cofactors and Vitamins | Retinol metabolism	2.1(1.8-2.5)	2.2(2.0-2.5)	0.3813	0.9969
Metabolism | Metabolism of Cofactors and Vitamins | Riboflavin metabolism	1.8(1.6-2.3)	1.9(1.6-2.3)	0.0502	1.0000
Metabolism | Metabolism of Cofactors and Vitamins | Thiamine metabolism	0.2(0.0-0.2)	0.2(0.1-0.2)	0.5072	0.9916
Metabolism | Metabolism of Cofactors and Vitamins | Ubiquinone and other terpenoid-quinone biosynthesis	0.2(0.1-0.4)	0.3(0.2-0.4)	0.3395	0.9956
Metabolism | Metabolism of Cofactors and Vitamins | Vitamin B6 metabolism	0.1(0.0-0.2)	0.2(0.1-0.3)	1.0000	1.0000
Metabolism | Metabolism of Other Amino Acids | beta-Alanine metabolism	0.4(0.2-0.6)	0.4(0.2-0.6)	0.7079	1.0000
Metabolism | Metabolism of Other Amino Acids | Cyanoamino acid metabolism	0.1(0.0-0.2)	0.1(0.0-0.2)	0.0662	1.0000
Metabolism | Metabolism of Other Amino Acids | D-Alanine metabolism	0.1(0.0-0.1)	0.1(0.0-0.1)	0.6458	1.0000
Metabolism | Metabolism of Other Amino Acids | D-Arginine and D-ornithine metabolism	0.4(0.3-0.4)	0.3(0.2-0.4)	0.1033	1.0000
Metabolism | Metabolism of Other Amino Acids | D-Glutamine and D-glutamate metabolism	0.1(0.0-0.1)	0.0(0.0-0.1)	0.0843	1.0000
Metabolism | Metabolism of Other Amino Acids | Glutathione metabolism	0.1(0.0-0.2)	0.0(0.0-0.1)	0.6456	1.0000
Metabolism | Metabolism of Other Amino Acids | Phosphonate and phosphinate metabolism	0.3(0.2-0.4)	0.3(0.2-0.4)	0.0642	1.0000
Metabolism | Metabolism of Other Amino Acids | Selenocompound metabolism	0.1(0.0-0.2)	0.1(0.1-0.2)	0.1325	0.9915
Metabolism | Metabolism of Other Amino Acids | Taurine and hypotaurine metabolism	0.1(0.0-0.2)	0.1(0.0-0.2)	0.5015	0.9984
Metabolism | Metabolism of Terpenoids and Polyketides | Biosynthesis of ansamycins	0.1(0.0-0.2)	0.1(0.0-0.1)	0.8659	1.0000
Metabolism | Metabolism of Terpenoids and Polyketides | Biosynthesis of siderophore group nonribosomal peptides	0.1(0.0-0.1)	0.1(0.0-0.2)	**0.0350**	1.0000

Table 10. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Metabolism | Metabolism of Terpenoids and Polyketides | Biosynthesis of vancomycin group antibiotics	0.1(0.0-0.2)	0.1(0.0-0.1)	0.9313	1.0000
Metabolism | Metabolism of Terpenoids and Polyketides | Geraniol degradation	0.0(0.0-0.1)	0.1(0.0-0.1)	0.2864	1.0000
Metabolism | Metabolism of Terpenoids and Polyketides | Limonene and pinene degradation	0.2(0.1-0.2)	0.2(0.1-0.4)	0.7392	1.0000
Metabolism | Metabolism of Terpenoids and Polyketides | Polyketide sugar unit biosynthesis	0.0(0.0-0.1)	0.0(0.0-0.1)	0.8672	1.0000
Metabolism | Metabolism of Terpenoids and Polyketides | Prenyltransferases	0.1(0.0-0.1)	0.0(0.0-0.1)	0.3527	0.9940
Metabolism | Metabolism of Terpenoids and Polyketides | Terpenoid backbone biosynthesis	0.0(0.0-0.1)	0.0(0.0-0.1)	0.9010	1.0000
Metabolism | Metabolism of Terpenoids and Polyketides | Tetracycline biosynthesis	0.1(0.1-0.3)	0.1(0.1-0.2)	0.1950	1.0000
Metabolism | Metabolism of Terpenoids and Polyketides | Zeatin biosynthesis	0.1(0.0-0.2)	0.1(0.0-0.2)	0.6413	1.0000
Metabolism | Nucleotide Metabolism | Purine metabolism	0.1(0.0-0.1)	0.1(0.0-0.2)	0.5085	0.9852
Metabolism | Nucleotide Metabolism | Pyrimidine metabolism	0.0(0.0-0.0)	0.0(0.0-0.0)	0.5897	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Aminobenzoate degradation	0.2(0.1-0.2)	0.2(0.1-0.2)	0.5304	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Atrazine degradation	0.4(0.2-0.5)	0.3(0.1-0.5)	0.4502	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Benzoate degradation	1.0(0.9-1.3)	1.1(0.9-1.2)	0.2610	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Bisphenol degradation	0.1(0.1-0.3)	0.2(0.2-0.4)	0.6167	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Caprolactam degradation	0.1(0.1-0.2)	0.2(0.1-0.3)	0.8767	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Chloroalkane and chloroalkene degradation	0.5(0.2-0.6)	0.5(0.3-0.7)	0.8039	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Chlorocyclohexane and chlorobenzene degradation	1.2(1.0-1.5)	1.3(1.0-1.6)	0.2864	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Dioxin degradation	0.7(0.5-0.8)	0.6(0.4-1.0)	0.7694	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Drug metabolism - cytochrome P450	0.8(0.5-1.1)	0.7(0.6-1.0)	0.2237	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Drug metabolism - other enzymes	0.2(0.1-0.4)	0.1(0.1-0.2)	**0.0222**	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Ethylbenzene degradation	1.0(0.7-1.1)	0.8(0.7-1.1)	0.8662	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Metabolism of xenobiotics by cytochrome P450	0.4(0.3-0.5)	0.3(0.2-0.6)	0.3547	0.9868
Metabolism | Xenobiotics Biodegradation and Metabolism | Naphthalene degradation	0.0(0.0-0.1)	0.0(0.0-0.1)	0.6770	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Nitrotoluene degradation	0.1(0.1-0.3)	0.2(0.1-0.3)	0.0665	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Polycyclic aromatic hydrocarbon degradation	0.8(0.7-1.0)	0.8(0.5-1.1)	1.0000	1.0000

Table 10. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Metabolism | Xenobiotics Biodegradation and Metabolism | Styrene degradation	0.1(0.0-0.2)	0.1(0.0-0.1)	0.6695	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Toluene degradation	0.0(0.0-0.1)	0.1(0.0-0.1)	0.7815	1.0000
Metabolism | Xenobiotics Biodegradation and Metabolism | Xylene degradation	1.3(1.1-1.6)	1.3(1.0-1.5)	1.0000	1.0000
Organismal Systems | Digestive System | Bile secretion	1.1(0.8-1.2)	1.0(0.8-1.3)	0.4555	0.9786
Organismal Systems | Digestive System | Carbohydrate digestion and absorption	3.3(2.7-3.8)	3.2(3.0-3.6)	0.2796	1.0000
Organismal Systems | Digestive System | Mineral absorption	0.0(0.0-0.1)	0.0(0.0-0.6)	0.8767	1.0000
Organismal Systems | Endocrine System | Adipocytokine signaling pathway	0.0(0.0-0.1)	0.0(0.0-0.1)	1.0000	1.0000
Organismal Systems | Endocrine System | Insulin signaling pathway	0.0(0.0-0.0)	0.0(0.0-0.0)	0.8325	1.0000
Organismal Systems | Endocrine System | PPAR signaling pathway	0.0(0.0-0.0)	0.0(0.0-0.0)	0.8326	1.0000
Organismal Systems | Endocrine System | Progesterone-mediated oocyte maturation	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0533	1.0000
Organismal Systems | Environmental Adaptation | Plant-pathogen interaction	0.0(0.0-0.0)	0.0(0.0-0.0)	0.5038	0.9939
Organismal Systems | Excretory System | Proximal tubule bicarbonate reclamation	0.0(0.0-0.0)	0.0(0.0-0.0)	0.4363	1.0000
Organismal Systems | Immune System | Antigen processing and presentation	0.0(0.0-0.0)	0.0(0.0-0.0)	0.0857	1.0000
Organismal Systems | Immune System | NOD-like receptor signaling pathway	0.0(0.0-0.0)	0.0(0.0-0.1)	0.2998	0.9857
Organismal Systems | Immune System | RIG-I-like receptor signaling pathway	0.0(0.0-0.0)	0.0(0.0-0.0)	0.2864	0.9865
Organismal Systems | Nervous System | Glutamatergic synapse	0.0(0.0-0.0)	0.0(0.0-0.0)	0.8991	1.0000
Unclassified | Cellular Processes and Signaling | Cell division	0.0(0.0-0.0)	0.0(0.0-0.0)	0.4487	1.0000
Unclassified | Cellular Processes and Signaling | Cell motility and secretion	0.0(0.0-0.0)	0.0(0.0-0.1)	0.1443	1.0000
Unclassified | Cellular Processes and Signaling | Electron transfer carriers	0.0(0.0-0.0)	0.0(0.0-0.1)	0.6843	1.0000
Unclassified | Cellular Processes and Signaling | Germination	0.0(0.0-0.1)	0.0(0.0-0.1)	0.8267	1.0000
Unclassified | Cellular Processes and Signaling | Inorganic ion transport and metabolism	0.0(0.0-0.0)	0.0(0.0-0.1)	0.9666	1.0000
Unclassified | Cellular Processes and Signaling | Membrane and intracellular structural molecules	0.0(0.0-0.0)	0.0(0.0-0.0)	0.1060	1.0000
Unclassified | Cellular Processes and Signaling | Other ion-coupled transporters	0.0(0.0-0.0)	0.0(0.0-0.0)	0.9011	1.0000
Unclassified | Cellular Processes and Signaling | Other transporters	0.0(0.0-0.0)	0.0(0.0-0.0)	**0.0252**	1.0000
Unclassified | Cellular Processes and Signaling | Pores ion channels	0.0(0.0-0.0)	0.0(0.0-0.1)	0.1922	1.0000

Table 10. Continued.

	median (min-max) *in percent			
Functional gene categories	**Healthy**	**AD**	**M-W p-value**	**M-W FDR p-value**
Unclassified | Cellular Processes and Signaling | Signal transduction mechanisms	0.0(0.0-0.1)	0.0(0.0-0.2)	0.4552	1.0000
Unclassified | Cellular Processes and Signaling | Sporulation	0.0(0.0-0.0)	0.0(0.0-0.0)	0.6789	1.0000
Unclassified | Genetic Information Processing | Protein folding and associated processing	0.0(0.0-0.0)	0.0(0.0-0.0)	0.1706	1.0000
Unclassified | Genetic Information Processing | Replication, recombination and repair proteins	0.0(0.0-0.0)	0.0(0.0-0.0)	0.2622	1.0000
Unclassified | Genetic Information Processing | Restriction enzyme	0.0(0.0-0.0)	0.0(0.0-0.0)	**0.0084**	0.6076
Unclassified | Genetic Information Processing | Translation proteins	0.0(0.0-0.0)	0.0(0.0-0.0)	0.2131	1.0000
Unclassified | Metabolism | Amino acid metabolism	0.0(0.0-0.0)	0.0(0.0-0.0)	0.5332	0.9975
Unclassified | Metabolism | Biosynthesis and biodegradation of secondary metabolites	0.0(0.0-0.0)	0.0(0.0-0.0)	0.9652	1.0000
Unclassified | Metabolism | Carbohydrate metabolism	0.0(0.0-0.1)	0.0(0.0-0.1)	0.1233	1.0000
Unclassified | Metabolism | Energy metabolism	0.0(0.0-0.0)	0.0(0.0-0.0)	0.3200	1.0000
Unclassified | Metabolism | Glycan biosynthesis and metabolism	0.0(0.0-0.0)	0.0(0.0-0.0)	0.8767	1.0000
Unclassified | Metabolism | Lipid metabolism	0.0(0.0-0.0)	0.0(0.0-0.0)	0.3719	0.9842
Unclassified | Metabolism | Metabolism of cofactors and vitamins	0.0(0.0-0.1)	0.0(0.0-0.0)	0.0829	1.0000
Unclassified | Metabolism | Nucleotide metabolism	0.0(0.0-0.0)	0.0(0.0-0.0)	0.2864	0.9711
Unclassified | Metabolism | Others	0.0(0.0-0.0)	0.0(0.0-0.0)	0.9341	1.0000
Unclassified | Poorly Characterized | Function unknown	0.0(0.0-0.0)	0.0(0.0-0.0)	0.8355	1.0000
Unclassified | Poorly Characterized | General function prediction only	0.0(0.0-0.0)	0.0(0.0-0.0)	0.2635	1.0000

M-W p-value = P-value from Mann-Whitney U test comparing healthy dogs and dogs with AD.
M-W FDR p-value = P-value from Mann-Whitney U test that is adjusted based on the Benjamini and Hochberg False Discovery Rate.

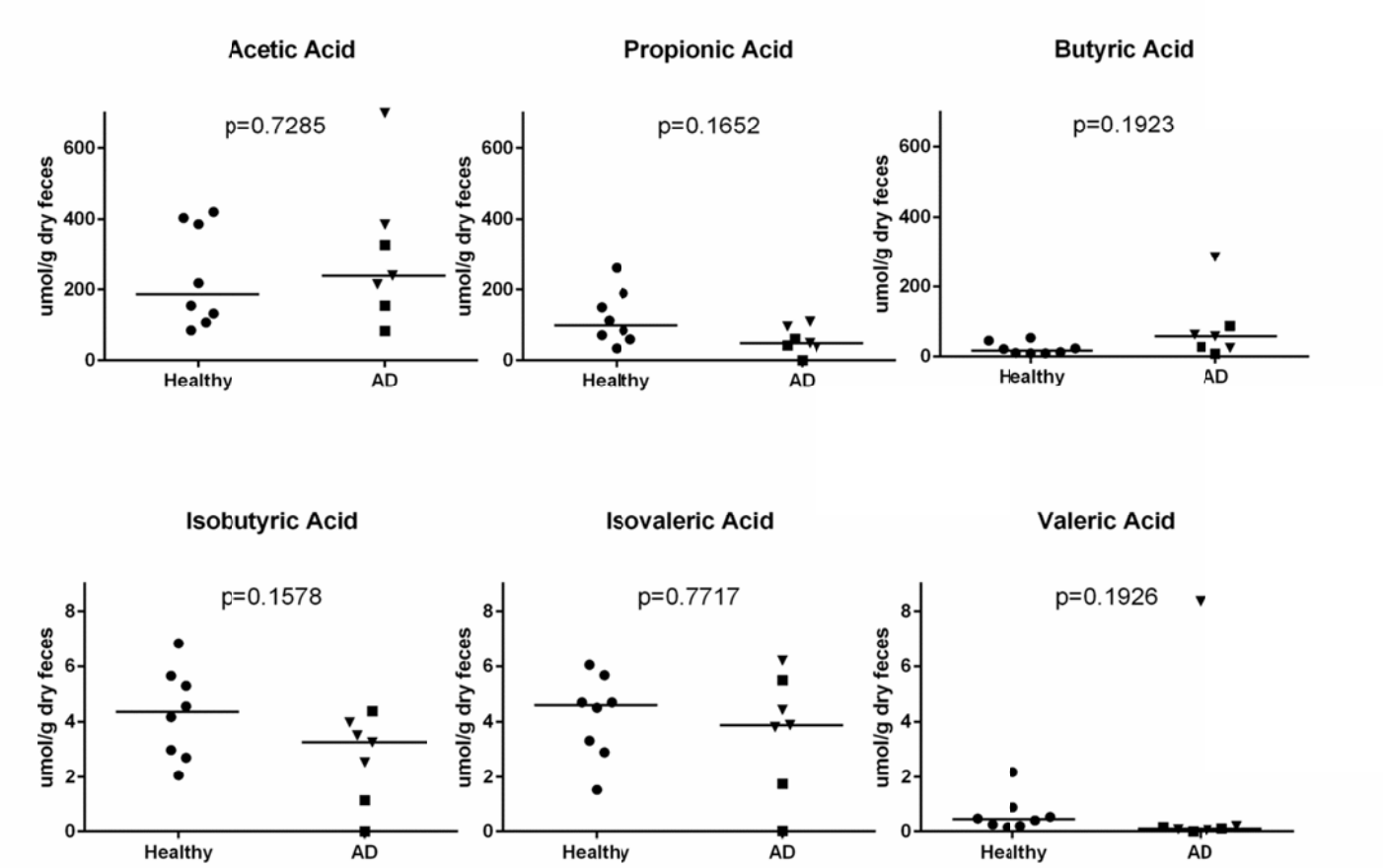

Figure 11. Total SCFA and BCFA concentrations (µmol/g dry feces). Circles ● = healthy dogs; Squares ■ = dogs with acute non-hemorrhagic diarrhea; Triangles▼ = dogs with acute hemorrhagic diarrhea. AD = both groups combined (NHD and AHD). Bars represent the median for each group.

Table 11. SCFA and BCFA concentrations.

	median (range) *μmol/g dry feces			
	Healthy	**AD**	**M-W p-value**	**M-W FDR p-value**
Acetic Acid	186.0(84.9-420.5)	238.8(83.1-700.0)	0.7285	0.7285
Propionic Acid	98.6(34.2-260.9)	48.7(0.2-109.5)	0.0826	0.1652
Butyric Acid	17.2(9.4-54.2)	58.2(8.7-285.1)	0.0641	0.1923
Isobutyric Acid	4.3(2.0-6.8)	3.2(0.0-4.3)	0.1052	0.1578
Isoveleric Acid	4.6(1.5-6.0)	3.8(0.0-6.2)	0.6431	0.7717
Valeric Acid	0.4(0.1-2.1)	0.1(0.0-8.3)	0.0321	0.1926

AD = both groups combined (dogs with acute non-hemorrhagic diarrhea and dogs with acute hemorrhagic diarrhea).
M-W p-value = P-value from Mann-Whitney U test comparing healthy dogs and dogs with AD.
M-W FDR p-value = P-value from Mann-Whitney U test that is adjusted based on the Benjamini and Hochberg False Discovery Rate.

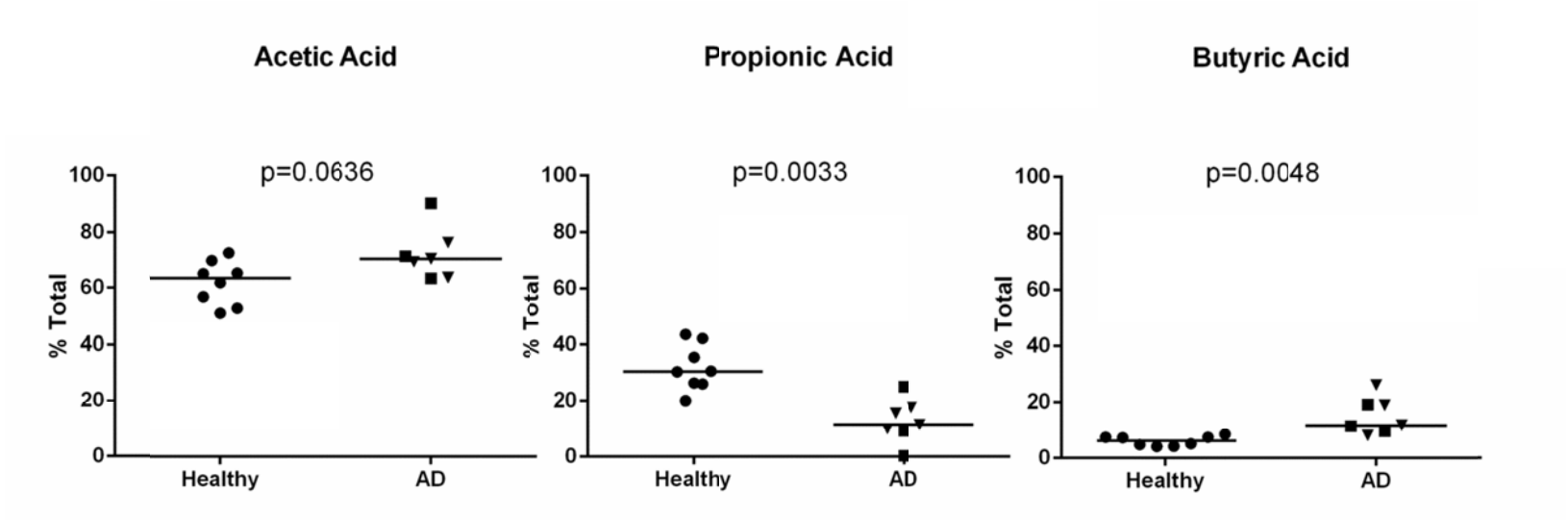

Figure 12. Proportions of specific SCFA of the total fecal SCFA concentrations. Circles ● = healthy dogs; Squares ■ = dogs with acute non-hemorrhagic diarrhea; Triangles ▼ = dogs with acute hemorrhagic diarrhea. AD = both groups combined (NHD and AHD). Bars represent the median value for each group.

Table 12. Proportions of specific SCFAs of the total fecal SCFAs concentration.

	median (range) *in percent			
	Healthy	**AD**	**M-W p-value**	**M-W FDR p-value**
Butyric acid	6.0(4.0-8.0)	12.0(8.0-26.0)	**0.0006**	**0.0048**
Propionic Acid	30.0(20.0-44.0)	12.0(0.0-25.0)	**0.0006**	**0.0033**
Acetic Acid	64.0(51.0-73.0)	71.0(64.0-90.0)	0.0721	0.0636

Healthy = healthy dogs; AD = both groups combined (NHD and AHD).
M-W p-value = P-value for Mann-Whitney U test comparing healthy dogs vs. dogs with AD.
M-W FDR p-value = P-value for Mann-Whitney U test that is adjusted based on the Benjamini and Hochberg False Discovery Rate.

DISCUSSION

The mammalian gastrointestinal tract (GIT) harbors a large number of prokaryotic organisms, mainly bacteria, which vastly exceed the number of host cells [83]. Molecular-based methods have been used previously to describe the intestinal microbiota of healthy dogs and have revealed a diverse and complex microbiota consisting of several hundred to several thousand phylotypes present in the GIT [4,59,60]. Additionally, bacteria are instrumental in the fermentation of carbohydrates and the production of SCFAs, which promote gastrointestinal health in the host [84].

Gastrointestinal disease (e.g., inflammatory bowel disease (IBD)) has been characterized by an altered composition of the intestinal microbiota in humans and also in dogs [3,59,60,85]. These studies observed a significantly decreased abundance of members of Bacteroidetes and Firmicutes and a significantly increased abundance of members of Proteobacteria [3,59,60,85].

Until recently, there have been limited studies that have described the fecal microbiota of dogs with acute GI disorders. In addition to this, limited information has been available regarding the relationship between phylogenetic differences, endogenous metabolites, and functional changes [69]. Therefore, for this study, we evaluated the fecal microbiome of healthy dogs, dogs with NHD, and dogs with AHD. We also evaluated the concentration of SCFAs and BCFAs. Furthermore, we predicted the functional genes of the microbiome based on the 16S rRNA gene data. Rarefaction curves (Figures 1, 2, and 3) and alpha diversity measures (Table 4) revealed a significant decrease in the Shannon Index for microbial diversity in dogs with AD compared to

healthy dogs. Similarly, trends were observed for a decreased number of observed species and the Chao1 metric in dogs with AD. However, this did not reach statistical significance. The reason for this could be due to the low number of samples analyzed in combination with the large inter-individual variation in microbial populations that was observed.

Results from this study are in general agreement with recent molecular studies that have examined the fecal microbiota of dogs with acute diarrhea. One study that investigated bacterial groups in the feces of dogs with acute diarrhea showed a significantly increased abundance of *Clostridium* spp. [56]. This study found that sequence percentages of *Clostridium* spp. were significantly increased in dogs with AD compared to healthy dogs (approximately a 2.5 fold increase); qPCR results from our study also showed a significant increase in the abundance of *Clostridium perfringens* in dogs with AD compared to healthy dogs (approximately a 2 log DNA increase). Studies in dogs with unspecified diarrhea showed significantly decreased proportions of Bacteroidetes compared to healthy dogs [54,63]. Similarly, our results showed significantly decreased proportions of Bacteroidetes compared to healthy dogs.

The 454-pyrosequencing results of this study showed a significant decrease of the abundance of *Faecalibacterium* spp. among dogs with acute diarrhea compared to healthy dogs. Studies in humans with IBD showed a decreased abundance of *Faecalibacterium prausnitzii* [86]. *Faecalibacterium prausnitzii* has been shown to secrete anti-inflammatory peptides in *in-vitro* studies [65]. More broadly, *Faecalibacterium* spp. have recently been suggested to constitute prominent members of

the canine GIT [87]. In one study, FISH analysis of fecal samples from healthy dogs identified that the *Faecalibacterium-Subdoligranulum* group comprised 16% of total bacterial counts [87].

SCFAs are well known to play a role in GI health and disease (e.g., colorectal cancer) [67]. Studies in humans have shown that colonic bacteria process fermentable carbohydrates to SCFAs, mainly acetate, propionate, and butyrate [67]. Phylogenetic groups within the phyla Firmicutes and Bacteroidetes (e.g., belonging to *Clostridium* clusters IV and XIVa), through the production of SCFAs, are thought to contribute commensal effects by regulation of the immune system and host metabolism (e.g., butyrate is known to decrease the permeability of the intestinal epithelial lining by increasing the expression of tight junction proteins and reinforcing colonic defense barriers by increasing antimicrobial peptide levels and mucin production [88,89]) [90]. In this study, the abundance of sequences belonging to *Faecalibacterium* and an unclassified genus within the family Ruminococcaceae were both decreased in dogs with AD compared to healthy dogs; qPCR results also showed a significant decrease in the abundance of *Blautia* spp. in dogs with AD compared to healthy dogs. Some of these groups are believed to be important producers of SCFAs [54,91,92]. Results from this study showed that the second most abundant SCFA in the feces, being propionic acid, was significantly decreased in dogs with AD compared to healthy dogs; this may correlate with a decrease in the aforementioned SCFA producing bacteria [93]. A study in obese humans found that genera like *Roseburia* spp., *Eubacterium rectale* (a subgroup of *Clostridium* cluster XIVa species), and *Bifidobacterium* (all thought to be butyrate

producing bacteria) were decreased as carbohydrate intake decreased and these butyrate producing bacteria correlated well with the decline in fecal butyrate [94]; these results show that a correlation may exist between SCFA concentrations and SCFA producing bacteria in feces.

In this study, the functional gene content of the microbiome was predicted from 16S rRNA gene data. Analysis of the relative abundance of broad functional categories of genes (e.g., KEGG derived metabolic pathways) revealed a generally consistent pattern regardless of the group of dogs (Figure 10B). This consistency is not only due to the broad level of functional categories, as broad taxonomic levels (e.g., phylum) revealed substantial variation across fecal microbiomes of all groups of dogs. Based on data from this study, it appears that a core fecal microbiome exists at the level of shared functional genes. The idea of a core microbiome is similar to what has been described in the literature in sequenced metagenomes of humans and is mirrored in the predicted gene content generated by the online software, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [95,96]. Some significant differences were identified based on the Mann-Whitney U test prior to correction by the Benjamini and Hochberg false discovery rate. Therefore, the following functional categories: xenobiotics biodegradation and metabolism, restriction enzymes (unclassified genetic information processing), sulfur relay system (genetic information processing: folding, sorting and degradation), glycosaminoglycan degradation (metabolism: glycan biosynthesis and metabolism), biosynthesis of siderophore group nonribosomal peptides (metabolism: metabolism of terpenoids and polyketides), drug metabolism (metabolism:

xenobiotics biodegradation and metabolism), and transporters (unclassified: cellular processes and signaling) may deserve further investigation.

As a limitation to this study, only small numbers of animals were enrolled in various disease groups and in the healthy group, partly due to cost restrictions. Additionally, in some instances, only a limited amount of sample was available; therefore some samples could not be included for all analyses. Also, all dogs, while all living in Texas, were on different diets and housed in different environments that were not controlled. Differing environments may have an effect on GI microbiota. In addition, functional genes were assigned based on the predicted metagenome based of 16S rRNA gene data instead of actually sequencing the entire microbial genome.

CHAPTER III

CONCLUSIONS

Previous studies that have employed sequencing-based methods to describe the intestinal microbiota of healthy dogs have shown a highly complex intestinal ecosystem [3,4,59,60,97]. GI disease has been intensely studied in dogs and the current literature suggests that alterations in the fecal microbiota may play a role in its pathogenesis [56,60].

Results of this study revealed a bacterial dysbiosis in fecal samples of dogs with NHD and dogs with AHD. Microbial diversity was significantly decreased amongst dogs with AD compared to healthy dogs (e.g., Shannon Index), indicative of an altered GI microbiota associated with GI disease.

The abundance of Bacteroidetes was significantly decreased in dogs with AD compared to healthy dogs. Also, the abundance of bacterial groups (e.g., *Faecalibacterium* spp., an unclassified genus within the family Ruminococcaceae, and *Blautia* spp.) that are consistently depleted in mammalian GI disease and considered to be important short-chain fatty acid producers were also decreased in dogs with AD. These groups may have a significant impact on the luminal concentrations of SCFA. The abundances of bacteria associated with SCFA production, were decreased and this may correlate with a decrease in fecal propionate.

Culture-independent 16S rRNA gene-based techniques have greatly expanded our understanding of bacterial phylogeny. Up until recently, a metagenomics approach has been widely regarded as costly and therefore impractical, and while some studies

have taken this approach, the metagenome of feces in dogs remains largely unstudied. Our study observed a core microbiome at the level of shared functional genes.

Future studies should further correlate metagenomics and the functional gene content in dogs with acute diarrhea. A more in depth look at metabolites in dogs with acute diarrhea also deserves more attention to begin to understand pathways that are up- or down-regulated in the dogs with acute diarrhea. Also, SCFA concentrations and their relation to bacteria which produce SCFA should be analyzed in respect to their implications for dogs with acute diarrhea.

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